THE BIOCHEMICAL JOURNAL

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(5) whether the presentation is reasonably concise.

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CORRIGENDA

The reaction of tetrahydropteroylglutamic acid and related hydropteridines with formaldehyde

By R. L. Blakley

Volume 72 (1959)

p. 708, column 2, last line

for 112–116° *read* 212–216°

Steps in the oxidation of xanthine to uric acid catalysed by milk xanthine oxidase

By H. Gutfreund and J. M. Sturtevant

Volume 73 (1959)

p. 5, column 1, line 14

for 50 μM^{-1} sec.$^{-1}$ *read* 5×10^5 M^{-1} sec.$^{-1}$

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Studies in Detoxication

80. THE METABOLISM OF GLYCOLS*

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(*Received* 11 *June* 1959)

The glycols, particularly ethylene, propylene and hexylene glycols, are important industrial chemicals (see Curme & Johnston, 1953). Although many toxicological studies have been made on these compounds (see Browning, 1953), few of them have been investigated from a metabolic point of view. In this paper a general survey of the metabolic reactions of 22 glycols has been carried out. Some were of industrial importance, and others had been tested for anaesthetic properties (see Buttle & Bower, 1958).

The metabolic reactions of the glycols are likely to depend upon the occurrence of primary, secondary or tertiary alcoholic groups, and upon the spatial relationships of these groups, i.e. whether they are vicinal or separated. A possible reaction of all glycols is conjugation with glucuronic acid. Other expected metabolites are hydroxymonocarboxylic acids, dicarboxylic acids and, in some cases, ketones. It has been reported that ethylene glycol does not increase the glucuronic acid excretion of rabbits (Miura, 1911) even after large doses (4 ml./kg.) (Fellows, Luduena & Hanzlik, 1947). A small glucuronic acid conjugation in rabbits has been reported for propane-1:2-diol (Neubauer, 1901; Miura, 1911; Fellows *et al.* 1947). Glucuronides are formed from butane-2:3-diol (Westerfeld & Berg, 1943) and 2:3-dimethylbutane-2:3-diol (pinacol) (Thierfelder & Mering, 1885) in rabbits, and from the anticonvulsant 2:2-diethylpropane-1:3-diol, in man (Berger & Ludwig, 1950).

EXPERIMENTAL

Animals. Chinchilla rabbits (2–3 kg. wt.) were kept on a constant diet of 60 g. of rat cubes (diet 41; Associated London Flour Millers) and 100 ml. of water per day. Glycols were administered by stomach tube with water.

Analytical method. Urine was analysed daily for glucuronic acid by the naphtharesorcinol method of Paul (see Mead, Smith & Williams, 1958).

Materials. Ethane-1:2-diol, propane-1:2- and -1:3-diol, butane-1:3-, -1:4- and -2:3-diol, pentane-1:5-diol, 2-methylpentane-1:4-diol, 3-methylpentane-1:5-diol, hexane-1:6-diol and 2:3-dimethylbutane-2:3-diol were purchased and purified. Butane 1:2-diol was synthesized (Matignon, Moureu & Dodé, 1935). Heptane-1:3-diol, 2-ethyl-, 2:2-dimethyl- and 2:2-diethyl-propane-1:3-diol, 2-methyl-2-*n*-propylpropane-1:3-diol, 2-methyl- and 2-ethyl-pentane-1:3-diol, 2-ethylhexane-1:3-diol, 2-ethylheptane-1:3-diol and 2-methylpentane-2:4-diol were presented by Professor G. A. H. Buttle and by Mr E. E. Connolly of The Distillers Co. Ltd. (Chemical Division), Hull (see also Buttle & Bower, 1958). Boiling points and melting points were checked with the literature.

Metabolites of ωω′-diols

Isolation of metabolites. The glycols diluted with water were administered orally to rabbits at a dose level of 1·0–1·5 g./kg. Urines were collected from the animals usually for 1–2 days after dosing and were acidified to a pH of about 2 by the addition of conc. HCl. They were then continuously extracted with ether for 16 hr. The ether extracts were evaporated and the residues dissolved in 2N-Na_2CO_3 (20 ml.). The alkaline solutions thus obtained were extracted with ether to remove non-acidic material, but only with 3-methylpentane-1:5-diol was any unchanged diol isolated from this fraction. The ether-extracted alkaline solutions were acidified with 2N-HCl and the acids present taken up with ether. On evaporation of this ethereal solution partly crystalline gummy residues of dicarboxylic acids were obtained, from which the acids were isolated by recrystallization or as suitable derivatives (see below and Table 1).

Propane-1:3-diol. A total of 16 g. of this diol was fed to four rabbits. Urines were collected for 3 days after dosing, but neither the unchanged diol nor malonic acid was isolated.

Butane-1:4-diol. From the urine of four rabbits given a total of 9 g. of this diol there was isolated 0·81 g. (7% of dose) of succinic acid, m.p. and mixed m.p. 189°, after recrystallization from water. After treatment with thionyl chloride, followed by aniline, the acid yielded succinanilide, m.p. and mixed m.p. 228°. No unchanged diol was found in the urine.

Pentane-1:5-diol. Four rabbits were given a total of 8·5 g. of this diol. The unchanged glycol was not excreted. The Na_2CO_3 solution of the extract from this urine was acidified with 2N-HCl, boiled to expel CO_2 and then neutralized with NH_3 soln. A saturated aqueous solution of $CuSO_4$ (10 ml.) was added and the mixture, which had become acid, was neutralized with NH_3 soln. A precipitate (0·7 g.) of copper salts separated and was filtered and washed with water, ethanol and finally ether. The precipitate was dissolved in 2N-HCl (20 ml.) and the solution extracted with ether. Evaporation of the ether left a gum (0·4 g.), which, on treatment with phenacyl bromide (1 g.)

* Part 79: Elliott, Parke & Williams (1959).

and recrystallization of the product from ethanol, gave phenacyl glutarate, m.p. and mixed m.p. 104° (yield 136 mg. or 0·5% of dose).

3-*Methylpentane*-1:5-*diol*. A total of 10 g. of the glycol (1·5 g./kg.) was fed to two rabbits. The ether extract of the acidified urine was extracted with 2N-Na_2CO_3 (3 × 20 ml.) to remove acids, and was then evaporated to dryness to leave a pale-brown liquid (1·45 g.). This was distilled (b.p. 224–225°) and the distillate was identified as 3-methylpentane-1:5-diol, n_D^{25}, 1·453 [Wojcik & Adkins (1933) give n_D^{25}, 1·452]. Treatment of the diol with 3:5-dinitrobenzoyl chloride gave the bis-3:5-dinitrobenzoate of 3-methylpentane-1:5-diol, m.p. and mixed m.p. 121° from aqueous ethanol (cf. Dische & Rittenberg, 1954). The Na_2CO_3 extract was now acidified and extracted with ether. Evaporation of the extract gave a partially crystalline solid (3·5 g.; 30% of the dose) which was dried over P_2O_5 *in vacuo* and then triturated with benzene to yield crystals (1·9 g.) which were identified as 3-methylglutaric acid, m.p. and mixed m.p. 87° after recrystallization from benzene. The acid was further identified as its anhydride (m.p. and mixed m.p. 45°) after refluxing with acetic anhydride and distilling (Darbishire & Thorpe, 1905), and as 3-methylglutaric monoanilide, m.p. and mixed m.p. 120°, after heating the anhydride with aniline in benzene solution.

Hexane-1:6-*diol*. The diol (2·8 g.) was fed to a rabbit. None of the unchanged diol was isolated from the 2-day urine. The carboxylic acid fraction of the urine in Na_2CO_3 was acidified with an excess of conc. HCl and kept overnight at 0°. A crystalline deposit (320 mg.; 9% of the dose) separated, which was purified by dissolution in 2N-Na_2CO_3 (charcoal) and precipitation with HCl. The crystals were identified as adipic acid, m.p. and mixed m.p. 152°, from which the *p*-nitrobenzyl ester, m.p. and mixed m.p. 106°, was prepared.

2-*Substituted propane*-1:3-*diols*

Crystalline metabolic products could not be isolated from the urine of rabbits receiving 2-ethyl- or 2:2-diethylpropane-1:3-diol, although Berger & Ludwig (1950) have shown that 2:2-diethylhydracrylic acid is a metabolite of the 2:2-diethyldiol in man.

2:2-*Dimethylpropane*-1:3-*diol*. This diol is highly conjugated (62%) with glucuronic acid in the rabbit (see Table 1). The glucuronide and its triacetyl methyl ester were readily obtained as gums by the lead acetate procedure (cf. Kamil, Smith & Williams, 1951), but these gums could not be crystallized.

The 24 hr. urine of four rabbits given a total of 8 g. of the diol was brought to pH 10·0 with NH_3 soln. and then continuously extracted with ether for 6 hr. Evaporation of the extract and sublimation of the residue yielded unchanged 2:2-dimethylpropane-1:3-diol, m.p. and mixed m.p. 127° (yield, 56 mg. or 0·7% of the dose). The residual urine was brought to pH 4·0 with 2N-HCl and again continuously extracted with ether for 6 hr. Evaporation of the extract gave an acidic gum which partly crystallized on keeping at 0° for a week. Repeated crystallization of this material from ethanol–ether mixtures gave white needles (150 mg.) of hydroxypivalic acid (3-hydroxy-2:2-dimethylpropionic acid), m.p. and mixed m.p. 126° (Found: equiv. 121. Calc. for $C_5H_{10}O_3$; equiv. 118). The authentic acid was prepared according to Wessely (1901).

2-*Methyl*-2-n-*propylpropane*-1:3-*diol*. Four rabbits were given a total of 32 g. of this diol, and the subsequent 24 hr. urine was continuously extracted with ether as described above, first after making alkaline with NH_3 soln. and again after making the residual urine acid. Neither of these extractions yielded crystalline products. The residual urine after the second extraction was therefore saturated with $(NH_4)_2SO_4$ and then extracted by shaking with a mixture of ethanol and ether (1:2). Evaporation of the solvents yielded a glucuronide gum (9·4 g.) which did not crystallize. The gum was therefore methylated with diazomethane and then acetylated with pyridine and acetic anhydride as described by Kamil *et al.* (1951), but again the triacetyl methyl ester was obtained as a gum. This gum was taken up in $CHCl_3$ and the solution washed three times with 2N-HCl and then thrice with saturated aq. $NaHCO_3$ solution. The washed solution, after drying over anhydrous Na_2SO_4, was evaporated to yield a gum (2·3 g.), which crystallized with considerable difficulty to yield 170 mg. of a compound, m.p. 116° and $[\alpha]_D^{20}$ – 25° in $CHCl_3$ (*c*, 1), which analysed as *methyl* (2-*acetoxymethyl*-2-*methyl*-n-*pentyl tri*-O-*acetylglucosid*)*uronate* (Found: C, 53·95; H, 6·9; CH_3CO, 35·9. $C_{22}H_{34}O_{12}$ requires C, 54·0; H, 6·8; CH_3CO, 35·2%).

The glucuronide gum (0·5 g.), which had been prepared by ethanol–ether extraction as described above from the 24 hr. urine of a rabbit dosed with 5 g. of the diol, was heated under reflux for 2 hr. with 5N-HCl (10 ml.). The resulting solution was extracted with ether, and the extract was dried over anhydrous Na_2SO_4 and then evaporated. The dark semi-crystalline residue was treated with a solution of phosgene in toluene according to Ludwig & Piech (1951) to form a carbamate, and the product was recrystallized from hot water. There was obtained 2-methyl-2-*n*-propylpropane-1:3-diol dicarbamate (meprobamate), m.p. and mixed m.p. 105°.

Other diols

Unsuccessful attempts were made to isolate crystalline glucuronides or other metabolites with propane-1:2-diol (of which 300 g. was fed in one experiment), 2-methyl- and 2-ethyl-pentane-1:3-diol, 2-methylpentane-1:4- and -2:4-diol, and 2:3-dimethylbutane-2:3-diol (pinacol). Most of these diols contain one or two asymmetric carbon atoms and therefore more than one glucuronide could be formed; this would tend to make the isolation of crystalline glucuronides difficult.

Butane-2:3-*diol*. This compound could be expected to give rise to at least two glucuronides of the diol itself, to acetoin and its glucuronides and to diacetyl. Neither diacetyl nor acetoin was detected (by spectra and colour tests; see Feigl, 1956) in the expired air or urine of rabbits dosed with 1·2–1·5 g. of this compound. However, a glucuronide was isolated from the urine of four rabbits which had been dosed with 2 g. each of the diol. The glucuronide gum was prepared by the lead acetate method and was methylated and acetylated in the usual manner. The triacetyl methyl ester was obtained in poor yield from ethanol as white needles of m.p. 151° and $[\alpha]_D^{20}$ – 18° in $CHCl_3$ (*c*, 1). It analysed as *methyl* (2-*hydroxybutyl*-3-*tri*-O-*acetylglucosid*)*uronate* (Found: C, 49·9; H, 6·2; CH_3CO, 32·4. $C_{17}H_{26}O_{11}$ requires C, 50·2; H, 6·45; CH_3CO, 31·8%).

Table 1. *Glucuronic acid conjugation and metabolites of glycols in rabbits*

The results for glucuronic acid conjugation are given as means for three animals with ranges in parentheses.

Glycol fed	Dose (m-moles/kg.)	Glucuronic acid conjugation (% of dose)	Urinary metabolites characterized
1. Glycol type: $(CH_2)_n \cdot (CH_2 \cdot OH)_2$			
Ethane-1:2-diol	4	0 (0)	CO_2 in expired air, traces of oxalate in urine
Propane-1:3-diol	4	1 (0–2)	None found
Butane-1:4-diol	4	1 (0–2)	Succinic acid
Pentane-1:5-diol	2	0 (0)	Glutaric acid
3-Methylpentane-1:5-diol	2	7 (3–11)	3-Methylglutaric acid
Hexane-1:6-diol	2	7 (4–9)	Adipic acid
2. Glycol type: $CRR' \cdot (CH_2 \cdot OH)_2$			
2-Ethylpropane-1:3-diol (R = H)	2	23 (18–28)	—
2:2-Dimethylpropane-1:3-diol	2	62 (53–67)	3-Hydroxy-2:2-dimethylpropionic acid
2:2-Diethylpropane-1:3-diol	2	35 (11–66)	—
2-Methyl-2-propylpropane-1:3-diol	2	33 (23–49)	Glucuronide of the glycol
3. Glycol type: $R \cdot CH(OH) \cdot CHR' \cdot CH_2 \cdot OH$			
Butane-1:3-diol (R′ = H)	4	1 (0–2)	None found
2-Methylpentane-1:3-diol	2	22 (21–23)	None isolated
2-Ethylpentane-1:3-diol	2	48 (46–52)	—
2-Ethylhexane-1:3-diol	2	7 (4–11)	None isolated
Heptane-1:3-diol (R′ = H)	2	9 (7–13)	—
2-Ethylheptane-1:3-diol	2	9 (8–10)	—
4. Glycol type: $R \cdot (CH \cdot OH)_2 \cdot R'$			
Propane-1:2-diol (R = H)	4	1 (0–3)	None found
Butane-1:2-diol (R = H)	2	12 (7–17)	None found
Butane-2:3-diol (R = R′ = CH_3)	4	22 (20–26)	Glucuronide of the glycol
5. Miscellaneous glycols			
2:3-Dimethylbutane-2:3-diol	2	27 (25–29)	Non-crystalline glucuronides not characterized
2-Methylpentane-2:4-diol	1	67 (49–93)[5]	
2-Methylpentane-1:4-diol	1·5	37 (32–43)[4]	

RESULTS AND DISCUSSION

The results of our experiments are summarized in Table 1. The first group of glycols have the general formula $CH_3(OH) \cdot [CH_2]_2 \cdot CH_2 \cdot OH$ and do not undergo glucuronic acid conjugation unless they contain six carbon atoms (i.e. 3-methylpentane-1:5-diol and hexane-1:6-diol), and even then the conjugation is less than 10% of the dose. The metabolic fate of ethane-1:2-diol has been briefly reported by Gessner & Williams (1959). The main metabolite of the ^{14}C-labelled compound in rats and rabbits is carbon dioxide. Oxidation to oxalic acid is a very minor reaction of the glycol, but rats and cats produce more oxalate than do rabbits and guinea pigs. Ethane-1:2-diol is oxidized in liver slices to glycolaldehyde and is then converted into carbon dioxide (Gessner, 1958). No malonic acid was detected in the urine of rabbits receiving propane-1:3-diol even with doses of 4 g./kg. Three oxidation products of propane-1:3-diol are possible, β-hydroxypropionic aldehyde, β-hydroxypropionic acid (hydracrylic acid) and malonic acid. Malonic acid can apparently be metabolized through the tricarboxylic acid cycle (Lee & Lifson, 1951) and recently malonyl-coenzyme A has been found to be an intermediate in fatty acid synthesis (Brady, 1958; Gibson, Titchener & Wakel, 1958). Hydracrylic acid has also been reported to be metabolized in animal tissues (Green, Dewan & Leloir, 1937). It appears probable that propane-1:3-diol is oxidized completely to carbon dioxide in the body.

Butane-1:4-diol appears to be metabolized via succinic acid, since we were able to isolate this acid (yield, 7% of the dose) from the urine of rabbits given 0·75 g. of the diol/kg. Succinic acid is readily metabolized unless administered in large doses (Weitzel, 1947*a*, *b*; Emmrich, Neumann & Emmrich-Glaser, 1941). Similarly, pentane-1:5-diol appears to be metabolized via glutaric acid, since we were able to isolate small amounts of glutaric acid (0·5% of the dose of 0·75 g./kg.) from the urine after feeding with the glycol. Glutaric acid is known to be readily metabolized to carbon dioxide (Hobbs & Koeppe, 1958; Rothstein & Miller, 1952). As much as 30% of the dose (1·5 g./kg.) of 3-methylpentane-1:5-diol was isolated from the urine as 3-methylglutaric acid. It appears that the

3-methyl group hinders the further oxidation of the dicarboxylic acid. Our results suggest that the main outlines of the metabolism of these $\omega\omega'$-diols are:

$$(CH_2)_n(CH_2\cdot OH)_2 \rightarrow \begin{matrix}\text{intermediate}\\ \text{oxidation}\\ \text{products}\end{matrix} \rightarrow (CH_2)_n(CO_2H)_2 \rightarrow \begin{matrix}CO_2\\ \nearrow\end{matrix}$$

The second group of glycols in Table 1 are 2-substituted propane-1:3-diols, which are diprimary alcohols. These compounds are of interest for a number of them possess anticonvulsant and tranquillizing properties. In this group, however, glucuronic acid conjugation is appreciable, and dicarboxylic acids (i.e. substituted malonic acids) were not found as metabolites. Berger & Ludwig (1950) isolated 2:2-diethyl-3-hydroxypropionic acid as a metabolite of 2:2-diethylpropane-1:3-diol in man, but found no diethylmalonic acid. We have isolated 3-hydroxy-2:2-dimethylpropionic acid (hydroxypivalic or 2:2-dimethylhydracrylic acid) as a metabolite of 2:2-dimethylpropane-1:3-diol, and we also obtained the glucuronide of 2-methyl-2-propylpropane-1:3-diol. These results suggest that the main metabolic routes of this type of glycol are:

$$\begin{matrix}CH_2\cdot OH\\ |\\ C\cdot R\cdot R'\\ |\\ CH_2\cdot OH\end{matrix} \rightarrow \begin{matrix}CH_2\cdot O\cdot C_6H_9O_6\\ |\\ C\cdot R\cdot R'\\ |\\ CH_2\cdot OH\end{matrix} + \begin{matrix}CH_2\cdot OH\\ |\\ C\cdot R\cdot R'\\ |\\ CO_2H\end{matrix}$$

The third group of glycols in Table 1 are derivatives of propane-1:3-diol with one primary and one secondary alcohol group. Butane-1:3-diol is a compound of low toxicity. No metabolite of it is known and none was found in this work; it is probable that it is completely oxidized in the body via β-hydroxybutyric acid, which should be its primary oxidation product. 2-Methylpentane-1:3-diol and 2-ethylhexane-1:3-diol were also examined in some detail, but no recognizable metabolic product was isolated. The other glycols in this group were available only in small quantities and detailed examination of their metabolites was not possible. At present we have no clear idea of the metabolic fate of this type of glycol.

The fourth group of glycols are derivatives of propane-1:2-diol. No urinary metabolites of propane-1:2-diol or of butane-1:2-diol were isolated. Propane-1:2-diol is relatively non-toxic (its LD_{50} for rats is about 26 g./kg.; Smyth, Seaton & Fischer, 1941) and it is probably oxidized to lactic acid, through which it can enter normal metabolic processes; it is, in fact, converted into glycogen in rats (Rudney, 1950). Butane-2:3-diol is not oxidized to butane-2:3-dione (diacetyl) in dogs (Westerfeld & Berg, 1943) and we have found no diacetyl in the urine or expired air of rabbits dosed with the glycol. Neuberg & Gottschalk (1925) reported that it formed a glucuronide in rabbits. We have isolated this glucuronide as a triacetyl methyl ester and from elementary analysis it appears to be a monoglucuronide. Since butane-2:3-diol contains two asymmetric carbon atoms, monoglucuronides corresponding to the *dextro-*, *laevo-* and *meso*-glycols are possible.

The three glycols 2:3-dimethylbutane-2:3-diol (pinacol), 2-methyl-pentane-2;4-diol and 2-methylpentane-1:4-diol are conjugated with glucuronic acid to an appreciable extent. None of these glucuronides was isolated in a crystalline form.

SUMMARY

1. The fate of oral doses of 22 glycols has been investigated in the rabbit.

2. Glycols of the general formula,

$$CH_2(OH)\cdot[CH_2]_n\cdot CH_2\cdot OH,$$

where $n = 0$–6, do not form appreciable amounts of conjugated glucuronic acid. Ethane-1:2-diol is mainly oxidized to carbon dioxide. Butane-1:4-diol, pentane-1:5-diol and hexane-1:6-diol yield small amounts of the corresponding dicarboxylic acids in the urine, but appear to be mainly destroyed *in vivo*. 3-Methylpentane-1:5-diol yields large amounts of 3-methylglutaric acid in the urine.

3. Glycols of the general formula

$$CH_2(OH)\cdot CRR'\cdot CH_2\cdot OH$$

form appreciable amounts of conjugated glucuronic acid. These appear to be glucuronides of the unchanged diols, for the monoglucuronide of 2-methyl-2-*n*-propylpropane-1:3-diol was isolated. These glycols do not form dicarboxylic acids *in vivo*, but monocarboxylic acids. 3-Hydroxy-2:2-dimethylpropionic acid was isolated as a metabolite of 2:2-dimethylpropane-1:3-diol.

4. No definite information about the other glycols in Table 1 was obtained, except that some are highly conjugated with glucuronic acid (2-methyl- and 2-ethyl-pentane-1:3-diol, butane-2:3-diol, 2:3-dimethylbutane-2:3-diol, and 2-methylpentane-1:4- and -2:4-diol). The monoglucuronide of butane-2:3-diol was isolated.

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Studies in Detoxication

81. THE METABOLISM OF HALOGENOBENZENES: (*a*) PENTA- AND HEXA-CHLOROBENZENES. (*b*) FURTHER OBSERVATIONS ON 1:3:5-TRICHLOROBENZENE

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The metabolic fate of mono-, di-, tri- and tetrachlorobenzene has already been reported (Smith, Spencer & Williams, 1950; Azouz, Parke & Williams, 1955; Parke & Williams, 1955; Jondorf, Parke & Williams, 1955, 1958). These studies suggested that the more chlorine the halogenated benzene contained, the less readily was it metabolized. To complete the series of chlorinated benzenes, an investigation of the fate of penta- and hexa-chlorobenzene in rabbits has been carried out. Pentachlorobenzene is only slightly altered *in vivo*, and hexachlorobenzene seems to be metabolically inert. Studies of these highly chlorinated benzenes have been carried out in connexion with work in this Laboratory on the metabolism of the highly chlorinated insecticides, dieldrin and aldrin, which also appear to be metabolically inert. Pentachlorobenzene does not have any important practical application, but hexachlorobenzene has limited use as a fungicidal agent for seeds.

Jondorf *et al.* (1955) were unable to account for the major portion of the dose of 1:3:5-trichlorobenzene given to rabbits. This compound has been re-investigated and it is now clear that it is not readily metabolized.

METHODS AND MATERIALS

Melting points are corrected.

Materials. 1:3:5-Trichlorobenzene was prepared from 2:4:6-trichloroaniline and carefully purified; it had m.p. 69° (m.p. values from 61° to 64° are quoted in the literature). Pentachlorobenzene, m.p. 88° (Holleman, 1920) and hexachlorobenzene, m.p. 230° (Silberrad, 1922) were prepared and carefully purified, the samples used being judged to be free of less-chlorinated benzenes by absorption spectra (see Table 1, and tables of spectra quoted by Jondorf *et al.* 1958). *p*-Chlorophenol, m.p. 42°, and its toluene-*p*-sulphonate, m.p. 71°, and pentachlorophenol, m.p. 191°, and its benzoate, m.p. 160°, were prepared as reference compounds.

Animals. Chinchilla doe rabbits, kept throughout on a diet of 80 g. of rat cubes (diet 41; Associated London Flour Millers) and 100 ml. of water/day, were used. The chlorinated benzenes were administered either by stomach tube as aqueous suspensions or subcutaneously as 10% (w/v) solutions in arachis oil. Urine was collected daily.

Analytical methods. Glucuronic acid, ethereal sulphate and mercapturic acid outputs in urine were determined by methods already described (e.g. Jondorf *et al.* 1958). Spectrophotometric determinations were made with a Unicam spectrophotometer (SP. 500).

Estimation of chlorinated phenols. 2:4:6-Trichlorophenol in urine was determined spectrophotometrically (see Jondorf *et al.* 1955). Total pentachlorophenol in urine was determined from the difference between the light absorption of acid and alkaline solutions of steam-distillates of urines previously hydrolysed in 5N-HCl as described by Azouz *et al.* (1955) for dichlorophenols. Free pentachlorophenol was similarly estimated from steam-distillates of urine adjusted to pH 2·0 with 2N-HCl. At the wavelength used (319 mμ) the ε value for pentachlorophenol is 5400 in 0·1N-NaOH and 1200 in 0·1N-HCl. The light-absorption maxima of pentachlorophenol were at 250 and 319 mμ (ε 9700 and 5400 respectively) in 0·1N-NaOH, and at 292, 296, 300 and 304 mμ (ε 3250, 3300, 3300 and 3400 respectively) in 0·1N-HCl.

Pentachlorophenol (5 mg./100 ml.) added to normal rabbit urine (50 ml.) and hydrolysed by refluxing for 3 hr. with an equal volume of conc. HCl was recovered quantitatively (103±5%) in 0·5 vol. of distillate. Distillation from aqueous solution at pH 2·0 gave recoveries of only 75±5% in 0·5 vol. of distillate; water equal to the distillate was therefore added to the flask and the steam-distillation repeated. Three distillations in this way gave a recovery of 100±5%. Therefore a single distillation was used with hydrolysed urines, and three consecutive distillations for unhydrolysed urines.

Estimation of chlorinated benzenes. (i) In expired air. The estimation of 1:3:5-tri-, penta- and hexa-chlorobenzene in expired air was carried out spectrophotometrically as described by Azouz, Parke & Williams (1952) for other halogenated benzenes. 1:3:5-Trichlorobenzene was determined from its absorption at 280 mμ, pentachlorobenzene at 298 mμ and hexachlorobenzene at 301 mμ. Monochlorobenzene appeared to be present in some experiments and it was estimated at 264 mμ, correction being made for the absorption due to any other chlorinated benzene present.

(ii) In faeces and tissues. The estimations were carried out spectrophotometrically as described by Jondorf *et al.* (1958) for tetrachlorobenzenes. The 'pelt' included all the attached subcutaneous fat and 'depot fat' comprised all the macroscopic fat tissue excepting that of the pelt. After injection of the chlorinated benzenes, the pelt and adjacent tissues within a radius of about 2 cm. of the site of injection were separately examined.

Chromatography. The R_F values and colour reactions of the chlorophenols relevant to this work are given in Table 2.

Table 1. *Absorption spectra of penta- and hexa-chlorobenzene*

Solvent	Ethanol		*n*-Hexane	
	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$	$\lambda_{max.}$ (mμ)	$\epsilon_{max.}$
Pentachlorobenzene	288	360	261	310
	298	350	274	245
			278	250
			289	310
			298	360
Hexachlorobenzene	291	245	~280	225
	301	210	291	260
			299	230

Isolation and detection of 1:3:5-*trichlorobenzene*

In expired air. Two rabbits received orally 0·5 g./kg. of the trichlorobenzene. The expired air was drawn through ethanol (see Jondorf *et al.* 1958) and the spectrum of the ethanol was examined periodically. During the first 4 days after dosing, maxima were found only at 264, 272 and 280 mμ, which corresponded to 1:3:5-trichlorobenzene. From the fifth to the ninth days maxima also appeared at 245, 251 and 258 mμ, suggesting the presence of monochlorobenzene (see Fig. 1). With 280 mμ for trichlorobenzene and 264 mμ for monochlorobenzene (correcting for the amount of trichlorobenzene absorption at 264 mμ), it was estimated that these two rabbits eliminated, in 9 days, 8·8 and 11·9% of the dose as 1:3:5-trichlorobenzene and 1·5 and 0·6% as monochlorobenzene.

Table 2. *R_F values and colour reactions on paper of certain chlorophenols*

R_F values are for descending chromatography on Whatman no. 1 paper in solvent *A* and *B* and on Whatman no. 4 paper treated with aq. 0·2N-Na_2CO_3 in solvent *C*. Solvents: *A*, benzene–acetic acid–water (1:1:2, by vol.), run for 6 hr.; *B*, ethanol–butan-1-ol–aq. 6N-NH_3 soln.–6N-$(NH_4)_2CO_3$ (22:80:19:19, by vol.) (cf. Fewster & Hall, 1951) run for 12 hr.; *C*, *n*-hexane–di*iso*propyl ether (9:1, v/v) run for 0·5 hr. Detecting reagents were: Gibb's reagent (2% ethanolic solution of 2:6-dichloroquinonechloroimide followed by 2N-Na_2CO_3); diazotized *p*-nitraniline, followed by 2N-Na_2CO_3; diazotized sulphanilic acid, followed by 2N-Na_2CO_3; aq. 1% $FeCl_3$, followed by saturated $NaHCO_3$; 5N-HNO_2 followed by aq. 2% urea after heating the dried paper for 2 min. at 120°, followed by a solution of 0·5 g. of tetramethyl-*p*-diaminodiphenylmethane (Tetrabase) and 2 g. of citric acid in 100 ml. of water (Feigl, 1954).

Phenol	R_F values in solvent			Colour reactions				
	A	*B*	*C*	Gibbs's reagent	Diazotized *p*-nitraniline	Diazotized sulphanilic acid	Ferric chloride	HNO_3–Tetrabase
4-Chlorophenol	0·78	0·91	0·84	Violet	Violet	Orange	None	None
2:4:6-Trichlorophenol	0·92	—	0·10	Blue	None	None	None	None
Pentachlorophenol	0·92	0·85	0·04	None	None	None	None	Blue
4-Chlorocatechol	0·33	—	0·06	Blue	—	—	Green, neutral Blue, $NaHCO_3$	None

In faeces and tissues. 1:3:5-Trichlorobenzene (3·5 mg.), m.p. 63°, was isolated by steam-distillation of the whole of the depot fat (excluding that attached to the pelt) of one rabbit which had been dosed orally. Steam-distillates of the faeces, gut contents and the liver were shown by spectra to contain both monochlorobenzene and 1:3:5-trichlorobenzene, but depot fat, the pelt and the remaining tissues contained only 1:3:5-trichlorobenzene.

In urine. Steam-distillates of hydrolysed urines from the first 3 days after dosing showed absorption peaks at 311 and 296 mμ in 0·1 N-NaOH and at 292, 285, 280 and 275 in 0·1 N-HCl. Those at 311 mμ (in NaOH) and 292 and 285 mμ (in HCl) predominate and correspond to 2:4:6-trichlorophenol; the others were minor, and were indicative of monochlorophenols. From the fourth to the ninth days the absorption peaks due to monochlorophenols became the most predominant. Paper chromatography confirmed these findings, for in the urine excreted from 4 to 9 days after dosing both 2:4:6-trichloro- and 4-chloro-phenol were detected. No 4-chlorophenol could be detected chromatographically in the urine of the first 3 days. Paper chromatography of the residues left after steam-distillation of the hydrolysed urines of 4–9 days revealed the presence of 4-chlorocatechol in amounts equivalent to a total of about 1% of the dose.

Isolation and detection of pentachlorobenzene

In expired air. The expired air of rabbits given oral doses of 0·5 g. of pentachlorobenzene/kg. was drawn through ethanol. After 12 hr. the absorption spectrum of the ethanol showed minor peaks at 268, 272, 276 and 281 mμ. These peaks increased in intensity and reached a maximum after 60 hr. and were still detectable at 84 hr. after dosing. There was practically no light absorption at and above 290 mμ (see Fig. 2). These observations suggested that neither penta- nor tetra-chlorobenzene was appearing in the expired air. The observed peaks suggested the presence of tri- and possibly di- and mono-chlorobenzene. When the pentachlorobenzene was injected no absorption peaks appeared in the ethanol until 48 hr. after dosing, and then peaks were observed at 254, 262, 270, 276 and 282 mμ. The exhaled chlorinated benzene was assumed to be 1:3:5-trichlorobenzene and, calculating from the absorption at 272 and 280 mμ, 9 and 21% of oral doses appeared in the breath in two experiments of 3 and 4 days' duration. After injection only 1% appeared in the breath.

In faeces and tissues. Pentachlorobenzene, m.p. and mixed m.p. 88°, was readily isolated from faeces and gut contents of rabbits given pentachlorobenzene orally and from the pelt and depot fat of animals injected subcutaneously with the compound. The spectra of hexane extracts of steam-distillates of the faeces and gut contents suggested the presence of less-chlorinated benzenes.

In urine. The urine of three rabbits given a total of 4·5 g. of pentachlorobenzene was collected for 5 days and made 5 N with respect to HCl by the addition of conc. HCl. It was boiled under reflux for 3 hr. and then steam-distilled. The spectra of the distillate showed peaks at 275 and 280 mμ in 0·1 N-HCl and 292 and 297 mμ in 0·1 N-NaOH. These suggest the presence of *p*-chlorophenol and

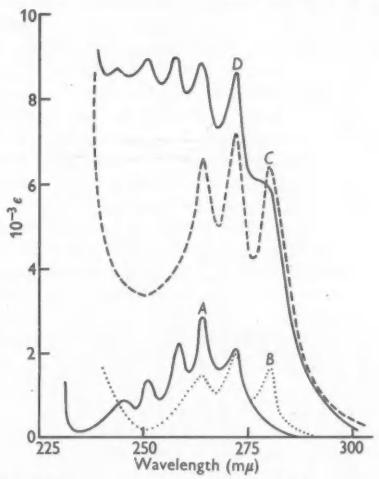

Fig. 1. Absorption spectrum in ethanol of the expired air of a rabbit after an oral dose of 1·5 g. of 1:3:5-trichlorobenzene compared with the spectra of chlorobenzene and 1:3:5-trichlorobenzene in ethanol. Ordinates for the curves of the expired air are the same but are arbitrary. Maxima (mμ): chlorobenzene (*A*), 245, 251, 258, 264, 272; 1:3:5-trichlorobenzene (*B*), —, —, —, 264, 272, 280; expired air, third day (*C*), —, —, —, 264, 272, 280; sixth day (*D*), 244, 251, 258, 264, 272, —.

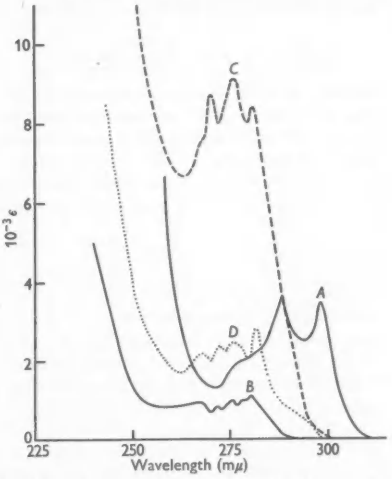

Fig. 2. Absorption spectrum in ethanol of the expired air of a rabbit after an oral dose of 1·3 g. of pentachlorobenzene compared with the spectrum of pentachlorobenzene in ethanol. Ordinates for the curves of the expired air are the same but are arbitrary. Maxima or inflexions (mμ): pentachlorobezene (*A*), —, —, —, —, —, 288, 298; expired air, first day (*B*), 268, 272, 276, 278, 281, —, —; third day (*C*), 268, 270, 276, —, 281, —, —; fourth day (*D*), 267, 272, 276, —, 282, —, —.

this was confirmed by paper chromatography. The distillates were extracted with ether, and the chlorophenols extracted were then transferred to N-NaOH. Treatment of the alkaline solution with toluene-*p*-sulphonyl chloride yielded 30 mg. of semi-solid material from which *p*-chlorophenyl toluene-*p*-sulphonate (3 mg.; m.p. and mixed m.p. 71°) was isolated. The ester on hydrolysis gave *p*-chlorophenol, which was identified chromatographically and by its spectrum in acid and alkali.

Isolation and detection of hexachlorobenzene

In expired air. Hexachlorobenzene (0·5 g./kg.) was administered orally to a rabbit and the expired air drawn through ethanol. No absorption peaks appeared in the ethanol during the 4 days of the experiment. When hexachlorobenzene was injected subcutaneously, no absorption peaks were found during the first 3 days, but on the fourth day insignificant peaks appeared at 245, 250, 255, 260, 265, 270, 278, 282 and 288 mμ.

In faeces and tissues. Hexachlorobenzene, m.p. and mixed m.p. 230°, was isolated from the faeces and gut contents of rabbits killed 5 days after being given hexachlorobenzene orally. It could also be isolated from the subcutaneous fat of rats injected with the compound. Steam-distillates of the gut contents of the rabbits fed with hexachlorobenzene were examined spectroscopically in hexane and very minor peaks were found at 278, 282 and 287 mμ in addition to the maxima corresponding to hexachlorobenzene. These might suggest the presence of 1:2:4:5-tetrachlorobenzene but the results were inconclusive. When hexachlorobenzene was injected, no chlorinated benzenes were found in the faeces after 5 days.

RESULTS AND DISCUSSION

A summary of the results obtained is given in Table 3, which shows that we have accounted for 75–85% of the chlorohydrocarbons administered. The three compounds do not undergo any extensive metabolic change *in vivo*, and it is doubtful whether hexachlorobenzene undergoes any change at all.

1:3:5-*Trichlorobenzene.* Jondorf *et al.* (1955) reported that this compound was oxidized to a small extent to 2:4:6-trichlorophenol. This has been confirmed. We have also obtained chromatographic evidence that 4-chlorophenol and 4-chlorocatechol are minor urinary metabolites. The formation of these phenols would suggest that the trichlorobenzene is partly dechlorinated to chlorobenzene, possibly by gut bacteria, and support for this was obtained on spectroscopic examination of the expired air (see Fig. 1). There was also evidence that chlorobenzene was present in the tissues. The exhalation of monochlorobenzene, however, only occurred 3–4 days after dosing with 1:3:5-trichlorobenzene. The main bulk of the administered trichlorobenzene was found unchanged in the tissues and gut contents 8–9 days after dosing (see Table 3).

Pentachlorobenzene. When given by mouth, the major portion of the dose of this compound is to be

Table 3. *Metabolism of* 1:3:5-*tri-, penta- and hexa-chlorobenzene in rabbits*

Doses were given orally unless indicated otherwise.

Compound	Dose (g./kg.)	Time after dosing (days)	Percentage of dose found in: Urine: Tri- or penta-chloro-phenol	Urine: Other phenols	Faeces	Gut contents	Pelt	Depot fat	Rest of body	Expired air: Unchanged	Expired air: Other chlorohydro-carbons	Total accounted for (%)
1:3:5-Trichlorobenzene	0·5	8	3	1	13	23*	5	5	22	12	0·6	85
	0·5	9	10	4	1·5	18	5	4·5	20	8·5	1·5	74
Pentachlorobenzene	0·5	3	0·2	1	5	45	1	15	6	0	9	82
	0·5	4	0·2	1	5	31	5	9	5·5	0	21	78
	0·5†	10	0·7	1	1·5	0·5	47‡	22‡	10	0	<2	85
Hexachlorobenzene	0·4	5	0	0	6	78	<1	<1	<1	0	<0·2	84
	0·4	5	0	0	6	73	1	1	2	0	<0·2	83
	0·1†	5	0	0	<0·3	0·2	75‡	<1	<1	0	<0·5	76

* 4% as monochlorobenzene. † Injected subcutaneously. ‡ Located mainly at site of injection.

found unchanged in the gut contents after 3–4 days. Some 20% is in the tissues generally and only about 5% in the faeces. When injected, the main bulk of the compound is found after 10 days in the tissues near the site of injection. On oral administration, some of the hydrocarbon (about 10%) is eliminated in a changed form in the expired air, and, from the spectrum of the expired air, this material is not unchanged pentachlorobenzene but a mixture of less-chlorinated benzenes. The suggestion that pentachlorobenzene is dechlorinated was supported by the isolation of *p*-chlorophenol in small amounts from the urine. The amounts of metabolites of pentachlorobenzene in the urine are very small, being not more than about 1% of the dose. In four experiments pentachlorophenol was detected in the urine with certainty in only two cases. The main urinary metabolites appear to be small amounts of *p*-chlorophenol and 4-chlorocatechol, of which the former was isolated.

Hexachlorobenzene. This compound does not appear to be metabolized, and when given orally the main bulk of the dose is to be found in the gut contents after 5 days, only 6% appearing in the faeces. There is no significant urinary or pulmonary excretion of metabolites. No attempt was made to prevent coprophagy by the animals used.

Output of conjugates after feeding with penta- and hexa-chlorobenzene. The urinary output of glucuronides, ethereal sulphates and mercapturic acids after the administration of pentachlorobenzene (0·5 g./kg.) and hexachlorobenzene (0·4 g./kg.) was determined in six animals for each compound. No significant rise in the output of these conjugates was observed, except for glucuronic acid after administering hexachlorobenzene, when an average increase in glucuronic acid output equivalent to 15% of the dose (range 9–23%) was observed. The increase appeared to be due to the excretion of free glucuronic acid, which was separated in the basic lead acetate fraction (Kamil, Smith & Williams, 1951) of the 2-day urine of two rabbits each dosed with 0·8 g. of hexachlorobenzene. The free glucuronic acid in this fraction was identified by colour reactions and its R_F, 0·18 in propan-1-ol–aq. NH_3 soln. (sp.gr. 0·88) (7:3, v/v) and 0 in butan-1-ol–aq. 3N-NH_3 soln. (2:3, v/v). It was concluded that penta- and hexa-chlorobenzene did not form conjugated glucuronic acids, ethereal sulphates or mercapturic acids.

SUMMARY

1. A study has been made of the fate in the rabbit of 1:3:5-tri-, penta- and hexa-chlorobenzene.

2. 1:3:5-Trichlorobenzene is not readily metabolized. The major portion of a dose of 0·5 g./kg. is unchanged in the gut contents and tissues 8 days after dosing. Some of the unchanged chlorohydrocarbon is eliminated in the faeces and expired air. A small proportion is converted into monochlorobenzene, some of which appears in the expired air (about 1% of the dose) and as *p*-chlorophenol in the urine. Less than 10% of the dose is excreted as 2:4:6-trichlorophenol.

3. Pentachlorobenzene is not readily metabolized. About 60% of the oral dose (0·5 g./kg.) is found in the gut contents and tissues 3–4 days after dosing. Some 10–20% is dechlorinated and eliminated in the expired air as less-chlorinated benzenes, and a small amount of *p*-chlorophenol was isolated from the urine, thus proving the occurrence of dechlorination. Injected pentachlorobenzene remains largely at the site of injection and was found there 10 days later. Pentachlorophenol was a very minor urinary metabolite ($< 0{\cdot}2$% of the dose).

4. It is doubtful whether hexachlorobenzene is metabolized at all. The major portion of oral doses (0·4 g./kg.) was found in the gut 5 days after dosing and most of an injected dose (0·1 g./kg.) was found at the site of injection after 5 days.

5. Pentachlorobenzene and hexachlorobenzene gave no ethereal sulphates, glucuronides or mercapturic acids.

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The Purification of the Enzyme Hydrolysing Diethyl *p*-Nitrophenyl Phosphate (Paraoxon) in Sheep Serum

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Aldridge (1953*b*) reported an enzyme in mammalian sera which hydrolysed the acetylcholinesterase inhibitor, diethyl *p*-nitrophenyl phosphate (paraoxon) to diethylphosphoric acid and *p*-nitrophenol. No serious attempts have previously been made to purify this enzyme either from serum or from other tissues. Aldridge (1953*b*) fractionated rabbit serum with ammonium sulphate, but none of the fractions showed significant purification. In addition he showed that the Cohn *et al.* (1950) method 10 precipitated the enzyme in the (I+II+III) fraction. The yield was 85% and purification 2·8-fold.

An enzyme hydrolysing a related substrate, di*iso*propylphosphorofluoridate, was purified 13-fold from rabbit kidney by Mazur (1946). Using a similar procedure, Mounter, Floyd & Chanutin (1953) purified hog-kidney di*iso*propyl phosphorofluoridatase 65–100 times. The most pure (A-2) fraction was shown by Cohen & Warringa (1957) to contain two and possibly three additional enzymes. Bergmann, Segal & Rimon (1957) have reported a 'C'-esterase from the A-2 fraction. 'C'-Esterase hydrolysed *p*-nitrophenyl acetate under essentially the same conditions as A-esterase, as defined by Aldridge (1953*a*). Aldridge had concluded that the enzyme hydrolysing paraoxon in serum was identical with A-esterase, which hydrolysed *p*-nitrophenyl acetate and was not inhibited by paraoxon. However, since an enzyme exists which does not hydrolyse paraoxon but does hydrolyse *p*-nitrophenyl acetate under the conditions defining an A-esterase, this identity may not hold in all cases. Evidence supporting the view that more than one paraoxon-resistant esterase hydrolyses *p*-nitrophenyl acetate in serum will be presented in a later paper dealing with the properties of sheep-serum paraoxonase. The activity hydrolysing paraoxon has been called paraoxonase to distinguish it from other A-esterases.

The relative contributions of these enzymes towards the hydrolysis of various substrates is difficult to assess in impure tissue homogenates. The situation is made more complex by the variable effects of metal ions with different substrates and tissues (Mounter & Chanutin, 1953, 1954) and by the possible presence of naturally occurring activators and inhibitors (Cohen & Warringa, 1957). The use of partially purified preparations has also given rise to uncertainties (Cohen & Warringa, 1957). The purpose of the present work was therefore to obtain a homogeneous preparation both to differentiate paraoxonase from enzymes with similar substrate specificities and, if possible, to gain some knowledge about the chemical nature and possible physiological function of this enzyme.

Since paraoxonase was a relatively labile enzyme, a purification procedure with mild fractionating conditions was desirable. The Cohn low-temperature ethanol-fractionation approach was satisfactory in this respect, but the procedures were not followed in detail. In the present work conditions were determined which simplified the Cohn *et al.* (1946) method 6 in the initial stages and at the same time increased both the yield and purification. In addition, it was desired to keep the dilution of the serum to a minimum to utilize the capacity of the refrigerated equipment to best advantage. In the later stages an independent study of the effect of ethanol, ionic strength and pH at low temperatures proved necessary.

EXPERIMENTAL

Colorimetric determination of paraoxonase activity. *p*-Nitrophenol in the paraoxon was removed by repeatedly shaking a 20% solution in $CHCl_3$ with a 1% $NaHCO_3$ soln. according to the method of Aldridge (1953*b*). A stock solution (0·8 M) was prepared by diluting 11·008 g. of *p*-nitrophenol-free paraoxon to 50 ml. with absolute methanol.

A buffered paraoxon substrate (8 mM) solution was prepared by blowing 1 ml. of 0·8 M-paraoxon stock solution into 100 ml. of M/15-sodium phosphate buffer, pH 7·6, containing 0·1% of gum acacia and by shaking vigorously. This reagent could be used for several days if stored in the cold.

A sample of buffered substrate (5 ml.) was pipetted into each of three or more 10 ml. volumetric flasks. If required, one flask was used for a reagent blank. The second served to correct for non-enzymic hydrolysis and the third flask was used to measure the *p*-nitrophenol liberated during incubation. After the solution in the second and third flasks had reached temperature equilibrium (37°), 0·02 ml. of the

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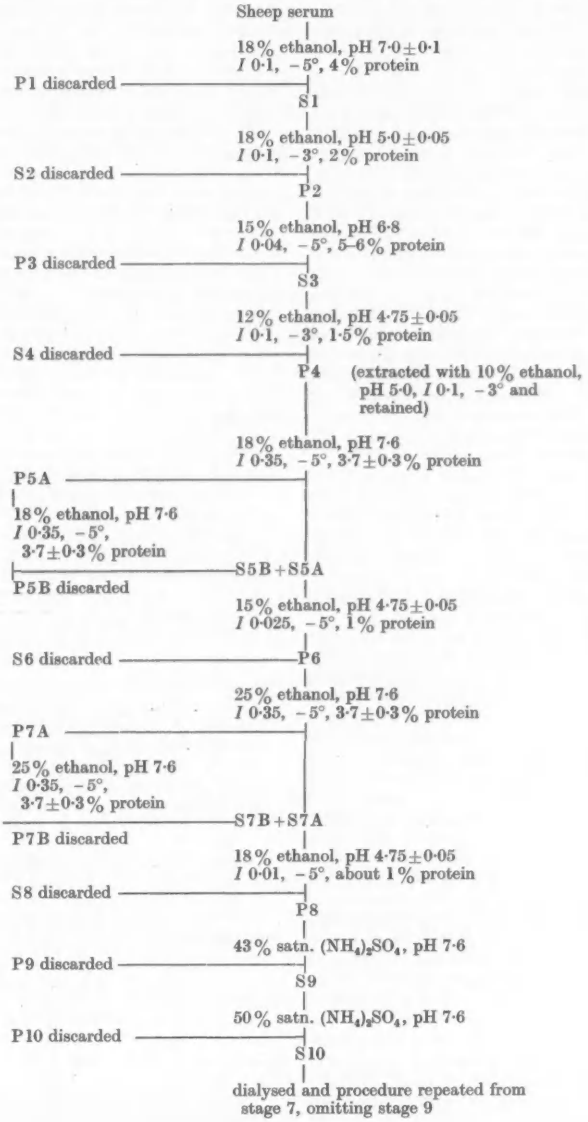
enzyme preparation was added to the third flask and the time was noted. After incubation for a suitable length of time, as judged visually by the colour in the flask, the third flask was removed from the bath and immediately made to volume with ethanol reagent (400 ml. of 95% ethanol and 6·25 ml. of aq. 2N-NH_3 soln., diluted to 500 ml. with water) to terminate enzymic hydrolysis and to bring the pH to 9·2. The time was again noted.

The pH was adjusted to 9·2 to prevent small pH fluctuations from affecting the extinction and to maintain the stability of paraoxon. Spontaneous paraoxon hydrolysis became rapid at pH 10·3, but was negligible in the region of

Flow scheme 1. Purification of serum paraoxonase. S, Supernatant; P, precipitate.



pH 9·2. The 400 mμ light absorption by *p*-nitrophenol increased from pH 6·0 to 8·5, but then became almost constant at higher pH.

The extinction was read at 400 mμ in cuvettes with a 1 cm. light path at any convenient time after the addition of ethanol reagent. The precision of the method was ±2·5%, with Sahli pipettes 'calibrated to contain' 0·02 ml. This volume of most enzyme preparations liberated a convenient amount of *p*-nitrophenol in 1–30 min. and thus eliminated the need for more than one dilution.

The *p*-nitrophenol calibration curve was made in the presence of 40% ethanol, since ethanol significantly altered the light absorption.

Manometric determination of paraoxonase. A variation of the manometric technique described by Aldridge (1953*a*) was also used to determine the activity against paraoxon and *p*-nitrophenyl acetate. Generally, 0·5 ml. of enzyme preparation was placed in the side arm of the manometer flask and 2·5 ml. of buffered substrate in the centre well. The final concentrations of the components in the digestion medium, exclusive of substrate and enzyme, were 25 mM-$NaHCO_3$, 0·162 M-NaCl and 0·2% of gelatin. The activities were determined at 37° after gassing for about 15 min. with $CO_2 + N_2$ (5:95) at room temperature.

Determination of protein. Routine protein determinations were made by measuring the light absorption at 280 mμ with a quartz u.v. spectrophotometer. In general a dilution of 0·02 ml. of sample in 4 ml. of 50 mM-$NaHCO_3$ soln. gave a convenient reading. The biuret method of Robinson & Hogden (1940) was also used to determine protein. Both methods were calibrated against the Kjeldahl method of Chibnall, Rees & Williams (1943). The spectrophotometric method at 280 mμ could be used for the earlier fractions from the procedure, but gave readings which were two to three times that of the Kjeldahl and biuret methods with the fractions including and following S7 (see flow scheme 1). The protein in these samples was routinely determined by the biuret method.

Phosphorus determination. Phosphorus was determined by the amidol method of Allen (1940).

Enzyme purification. Each stage of the purification procedure was associated with definite final precipitating conditions as given in the flow scheme. These conditions were determined by investigating the effect on the yield and purification of such variables as pH, alcohol concentration, ionic strength, time of standing after precipitation and rate and order of addition of reagents. Three examples illustrating the scope and nature of these investigations are given below. These examples also indicate the permissible limits of the specified variable necessary to obtain satisfactory purification and yields.

Sheep serum was selected as a source of paraoxonase since it was reasonably rich in enzyme and was readily available.

Stage 1. Defibrinated sheep blood (13 l.) was obtained from an abattoir on the day of slaughter and was used immediately. The fresh blood was centrifuged at 2000 *g* for 45 min. at room temperature. The serum was removed by suction and the volume measured. A volume convenient to the apparatus available was poured into a stainless-steel bucket and cooled in a bath at −5° until freezing just began. The remaining serum was stored at 0°.

To each litre of serum was added 500 ml. of reagent 1 (567 ml. of 95% ethanol and 17 ml. of 2 M-acetic acid mixed and diluted to 1 l. at room temperature) at the rate of 25 ml./min., with vigorous mechanical stirring. After standing overnight the mixture was centrifuged at 1800 *g* for 45 min. at −6°. The supernatant S1 was retained and the precipitate P1 was discarded. The supernatant volume was about 1380 ml./l. of serum.

Stage 2. S1 was adjusted to −3° and 25 ml. of 2 N-acetic acid/l. of serum was added, at the rate of 1·5 ml./min., with vigorous stirring. The pH was determined and, if necessary, adjusted to 5·0 ± 0·05. After standing for at least 12 hr. the solution was centrifuged at 1500 *g* for 30 min. at −3°. The supernatant S2 was discarded. The precipitate P2 was dissolved in 33 ml. of 0·1 M-$NaHCO_3$ soln./l. of serum and transferred with sufficient rinse water to make the final volume 125 ml./l. of serum.

The permissible limits of pH variation to obtain the highest yield and purification are shown in Fig. 1. In this example the limits were narrow, requiring careful pH control.

Table 1 illustrates the effect of standing time.

Stage 3. The pH of the dissolved precipitate P2 was adjusted to 6·8 with 2 N-acetic acid or aq. NH_3 soln. and cooled in a bath at −5° until ice crystals just appeared. Pre-cooled 95% ethanol (23 ml./125 ml. of P2) was added slowly with stirring. After standing overnight the solution

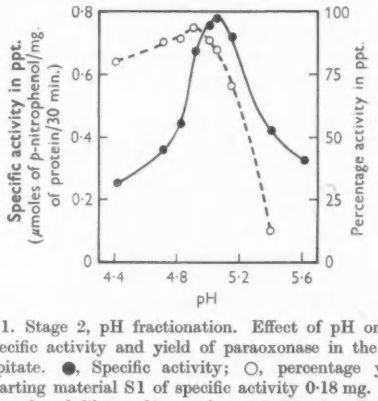


Fig. 1. Stage 2, pH fractionation. Effect of pH on the specific activity and yield of paraoxonase in the precipitate. ●, Specific activity; ○, percentage yield. Starting material S1 of specific activity 0·18 mg. of *p*-nitrophenol liberated/mg. of protein/30 min. Precipitating conditions: 18% (v/v) of ethanol; *I* 0·10; −3°; standing time for precipitate formation, 15 hr.

Table 1. *Rate of precipitation of paraoxonase from S1*

Starting material: S1; pH 5·0, *I* 0·1; ethanol 18%; temperature −3°.

Time of standing (hr.)	Activity in precipitate (%)	Activity in supernatant (%)
1	39	57
3	71	26
6	77	16
12	85	11
16	87	7

was centrifuged at 2000 *g* for 60 min. at −5°. The precipitate P3 was discarded. The supernatant S3, which was usually cloudy, was retained.

Stage 4. To each 100 ml. of S3 at −3° to −5° was added 8 ml. of 2M-NaCl solution and 87 ml. of water at room temperature. The diffuse precipitate cleared. The solution was placed in a cold bath at −3°.

A sample of convenient volume, usually 25 ml., was removed and titrated to pH 4·75 with 2N-acetic acid. Immediately after titration the sample was returned to the solution. The total volume of acid required to adjust the pH of S3 to 4·75 was calculated and was added with vigorous mechanical stirring at the rate of 2 ml./min. (about 2 ml. of acid/100 ml. of S3 was necessary).

To each initial 100 ml. of S3, now at pH 4·75, was added 9·5 ml. of pre-cooled 95% ethanol at the rate of 10 ml./min. After standing for 3 hr. at −3°, the solution was centrifuged at 1500 *g* for 30 min. at −3°. To collect the precipitate centrifuging was done with one 500 ml. centrifuge bottle and as many runs as necessary. The supernatant S4 was discarded.

The precipitate P4 was extracted at −3° with about 25 ml. of reagent 2 (105 ml. of 95% ethanol, 50 ml. of 2M-sodium acetate and 21 ml. of 2M-acetic acid, mixed and diluted to 1 l. pH 5·0)/100 ml. of S3. The extracting solution was stirred mechanically with the precipitate for about 30 min. at −3° and the mixture was centrifuged at 1500 *g* for 1 hr. at −3°. The supernatant was discarded.

The precipitate P4 was dissolved in 5 ml. of 0·1M-$NaHCO_3$ soln./100 ml. of S3 and rinsed into a measuring cylinder with an equal volume of water. Solubilization was aided by the use of a Potter homogenizer. The determination of the optimum ethanol concentration for the highest yield and purification in stage 4 is shown in Fig. 2.

Stage 5. The volume of the dissolved P4 was adjusted with water to 25 ml./l. of starting serum. A few drops of aq. 2N-NH_3 soln were added to adjust the pH to 7·6. To every 100 ml. of dissolved P4, 28 ml. of 2M-sodium acetate was added and the solution was warmed for 10 min. at 35°. After cooling the solution to 0°, 30 ml. of pre-cooled 95% ethanol/100 ml. of dissolved P4 was added slowly with stirring. During addition, the temperature was lowered to −5°. After standing overnight, the solution was centrifuged at 15 000 *g* for 60 min. at −5°.

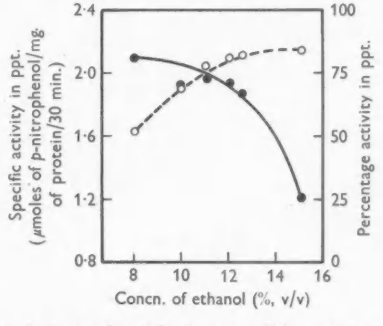


Fig. 2. Stage 4, ethanol fractionation. Effect of ethanol on the yield and purification of paraoxonase in the precipitate. ●, Specific activity; ○, percentage yield. Starting material, S3 of specific activity 0·91. Precipitating conditions: pH 4·75±0·05; *I* 0·10; temperature −5°; standing time for precipitate formation, 3 hr.

The supernatant, S5A, was dialysed overnight against 35 times its volume of cold mM-$NaHCO_3$ soln. and was then stored in the cold until needed. The precipitate P5A was dissolved in 12·5 ml. of water/l. of starting serum with the aid of a Potter homogenizer. The volume was adjusted to 20 ml./l. of serum. To every 100 ml. of dissolved P5A, 20 ml. of 2M-sodium acetate soln. was added. The solution was warmed at 35° for 10 min. After cooling the solution to 0°, 24 ml. of pre-cooled 95% ethanol was added slowly with stirring. During addition the temperature was lowered to −5°. After standing for 4 hr. the solution was centrifuged at 15 000 *g* for 60 min. at −5°. The precipitate P5B was discarded.

The supernatant S5B was dialysed overnight against 35 times its volume of cold mM-$NaHCO_3$ soln. The dialysed S5B was combined with the previous diffusate, S5A.

Stage 6. The volume of the combined diffusate, S5A + S5B, was measured. A 25 ml. sample was removed and titrated to pH 4·75 with 2N-acetic acid. The volume of acid required to adjust the pH of the diffusate to 4·75 was calculated. Usually about 1·4 ml. of acid/100 ml. of diffusate was necessary.

The diffusate was cooled to 0°, and 19 ml. of pre-cooled 95% ethanol and 1 ml. of 2M-NaCl soln./100 ml. of combined diffusate were added slowly with stirring. As the temperature of the solution was lowered to −5°, the volume of 2N-acetic acid calculated to bring the pH to 4·75 was added at the rate of 6 ml./min. After standing for 3 hr. the solution was centrifuged at 2000 *g* for 45 min. at −5°. The supernatant S6 was discarded.

The precipitate P6 was dissolved in 3 ml. of 0·1M-$NaHCO_3$ soln./100 ml. of combined diffusate. The dissolved P6 was transferred with a minimum of rinse water to a measuring cylinder.

Stage 7. The dissolved P6 was diluted with water to 12·5 ml./l. of starting serum and the pH was adjusted to 7·6 with a few drops of aq. NH_3 soln. Sodium acetate trihydrate (8 g./100 ml. of dissolved P6) was dissolved as the solution was warmed at 35° for 10 min. The solution was then cooled until it began to freeze and 33 ml. of 95% ethanol/100 ml. of P6 was added slowly, with stirring, as the temperature was lowered to −5°. After standing overnight the solution was centrifuged at 15 000 *g* for 60 min. at −5°.

The supernatant S7A was dialysed overnight against 50 times its volume of cold mM-$NaHCO_3$ soln. and then stored until needed. The precipitate P7A was dissolved in water and diluted to about 10 ml./l. of starting serum. To every 100 ml. of dissolved P7A was added 8 g. of sodium acetate trihydrate. The salt dissolved as the solution was warmed for 10 min. at 35°. The solution was cooled to 0°. As the temperature was lowered to −5°, 35 ml. of pre-cooled 95% ethanol/100 ml. of P7A was added slowly with stirring. After standing for 4 hr., the solution was centrifuged at 15 000 *g* for 60 min. at −5°. The precipitate P7B was discarded. The supernatant S7B was dialysed overnight against 50 times its volume of cold mM-$NaHCO_3$ soln. The diffusate S7B was combined with S7A.

Stage 8. The volume of the combined diffusates, S7A + S7B, was measured. A sample (25 ml.) was removed and

titrated to pH 4·75. The volume of acid required to adjust the pH of the diffusate to 4·75 was calculated. About 0·8 ml. of 2N-acetic acid/100 ml. of combined diffusate was usually required.

The diffusate was cooled to 0° and 24 ml. of pre-cooled 95% ethanol/100 ml. of combined diffusate was added slowly with stirring as the temperature was lowered to −5°. The calculated volume of 2N-acetic acid was then added at the rate of 3 ml./min. After standing for 1 hr., the solution was centrifuged at 12 000 *g* for 20 min. at −6°. The supernatant S8 was discarded. The precipitate P8 was dissolved in 1 ml. of 0·1M-$NaHCO_3$ soln./100 ml. of combined diffusate, and transferred with a minimum of rinsing water to a dialysis bag. The dissolved precipitate P8 was dialysed against 1 l. of cold mM-$NaHCO_3$ soln. for at least 6 hr.

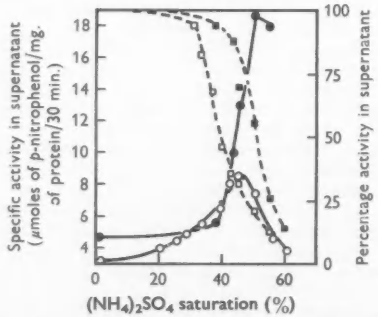


Fig. 3. Stages 9 and 10, ammonium sulphate fractionation. Effect of ammonium sulphate on the yield and purification of paraoxonase in the supernatant. ●, Specific activity, starting material, P6S; ○, specific activity, starting material, P4S; ■, percentage yield, starting material, P6S; □, percentage yield, starting material, P4S. Starting materials: P6S of specific activity 4·60 and P4S of specific activity 3·02 mg. of *p*-nitrophenol liberated/mg. of protein/30 min. Precipitating conditions: pH 7·6, adjusted with sodium phosphate buffer; 4°; standing time for precipitate formation, 1 hr.

Stage 9. The volume of dialysed P8 was measured and adjusted to 2–3 ml./l. of starting serum to keep the protein concentration at about 6%. Sufficient saturated $(NH_4)_2SO_4$ solution (7·5 ml./10 ml. of P8) was added with stirring at room temperature to make the solution 43% saturated. After standing for 4 hr. at about 4°, P8 was centrifuged at 15 000 *g* for 60 min. at 2°. The supernatant S9 was retained and the precipitate P9 was discarded (see Fig. 3).

Stage 10. The volume of S9 was measured and 1·5 ml. of saturated $(NH_4)_2SO_4$ soln./10 ml. of S9 was added to make the solution 50·5% saturated. After standing for 4 hr. at 4°, S9 was centrifuged at about 100 000 *g* for 30 min. at 4°. If a high-speed centrifuge was not available, centrifuging was done at 15 000 *g* for 2 hr. The precipitate P10, which was usually lighter than the supernatant S10, was discarded. When the volume of the precipitate was more than 20% of the total volume, the precipitate was washed with an equal volume of 50% saturated $(NH_4)_2SO_4$ soln. and recentrifuged. The supernatant of the wash was added to S10.

The supernatant S10 was retained and dialysed against cold mM-$NaHCO_3$ soln. for at least 12 hr. The dialysing solution was renewed once.

The term 'S10 repeated' in the text refers to a repetition of stage 10 on S10 after dialysis and re-concentration by stage 11.

Stage 11. After dialysis, S10 was treated as the combined diffusates, S7A+S7B, of stage 8. The supernatant S11 was discarded and the precipitate P11 was dissolved in 0·1M-$NaHCO_3$ soln. The solution was dialysed as in stage 8. If P11 was not clear or straw-coloured, stage 10 and stage 11 were repeated as noted above.

Efficiency of procedure. The results of each stage of the purification procedure are summarized in Table 2, which gives the results of run 31 for comparison with a composite run. Only those stages which resulted in significant purification are included.

The stages which are omitted served either to remove small amounts of interfering protein or to concentrate the enzyme. Of these, stage 3 precipitated 14·4±3·7% of the protein and 6·1±2·2% of the activity in P2. Only a small purification, of about 1·1 times, resulted in stage 3 but the purification obtained by stage 4 was much improved. Stages 6 and 8 concentrated the enzyme but did not result

Table 2. *Summary of the enzyme purification obtained in run* 31 *and in a composite run*

The composite run was constructed by averaging the results for each stage of a number of runs. Activities were determined by the colorimetric method. The specific activity is given in terms of μmoles of *p*-nitrophenol liberated/mg. of protein/30 min. at $V_{max.}$ and the absolute activity in terms of mg. of *p*-nitrophenol liberated/30 min. at $V_{max.}$.

	Run 31				Composite run			
Stage no.	Total activity	Specific activity	Yield (%)	Purification	No. of runs averaged	Specific activity	Yield (%)	Purification
Serum	11 000	0·093	100	1	16	0·095	100	1
1	9 650	0·166	88	1·8	9	0·176	86	1·85
2	9 140	0·78	83	8·4	15	0·76	81	8·0
4	7 060	2·81	64	30	8	2·70	63	27·5
5	5 470	5·25	50	58	9	4·75	48	50
7	3 780	11·8	34	127	5	10·8	32	114
10*	1 400	28·8	12·7	310	4	30·1	15	317

* Stages 9 and 10 in run 31 were repeated. The yield was lowered because of the incomplete removal of ethanol during dialysis.

in significant purification. The same precipitating conditions were used in stages 8 and 11 to concentrate the enzyme.

This procedure resulted in about 315-fold purification with a 15% yield up to stage 10. This material was usually slightly reddish but this colour could be removed by repeating stage 10. The purification then increased slightly to approx. 350±25 times and the yield decreased by about 20%.

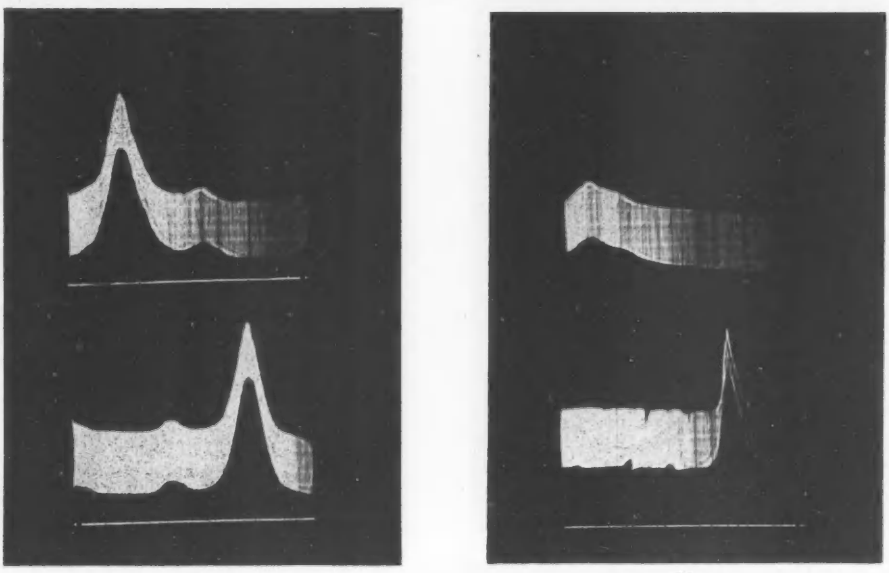
The performance of the individual stages is given in Table 3.

RESULTS AND DISCUSSION

Electrophoresis. Samples were subjected to electrophoresis in the analytical cell of the Perkin–Elmer model of the Tiselius boundary-electrophoresis apparatus. The schlieren diagrams of the purest preparations from seven runs showed the presence of two components. The leading component (Fig. 4*a*) was present in a higher concentration than the rear one. The leading boundary of the material shown in Fig. 5 was permitted to pass out of the descending limb, leaving only material represented by the rear boundary. The cell components were then isolated and the contents analysed. The material in the descending limb contained no activity towards paraoxon. It was water-clear and showed no fluorescence, whereas the starting material had been slightly cloudy and highly fluorescent. The material in the descending limb was about 0·04% of protein in a volume of 2·2 ml. This represented about 0·9 mg. of protein and is approximately the amount expected, considering the relative areas under the two peaks, the percentage of the material which remained in the descending limb after electrophoresis for 150 min. and the initial protein concentration. Analysis of the material in the bottom limb and in the ascending limb showed a specific activity, recalculated to $V_{max.}$, of 23·6 and 27·0 μmoles of *p*-nitrophenol liberated/mg. of protein/30 min. respectively. This

Table 3. *Summary of the yield of enzyme and purification obtained for each stage from a number of large-scale preparations*

Stage no.	No. of runs averaged	Purification from stage	Yield on stage (%)
1	9	1·9±0·2	86±2
2	16	4·3±0·5	94±3
4	8	3·4±0·4	79±4
5	9	1·8±0·2	76±2
7	5	2·4±0·2	67±10
9, 10	4	2·8±0·2	47±9



(*a*) (*b*)

Fig. 4. Analytical boundary electrophoresis; schlieren diagrams of 365-fold purified product at two pH values. (*a*) pH 8·5; top, ascending limb; bottom, descending limb; 0·1 M-veronal buffer; 105 V, 5·5 mA; time, 120 min. (*b*) pH 5·4; descending limb, top 45 min., bottom 165 min.; 0·01 M-sodium acetate, 0·09 M-NaCl; 100 V, 10 mA.

represented a purification of 307 times in the ascending limb. The contents of these two limbs were highly fluorescent and slightly cloudy. It was concluded that the material represented by the leading boundary contained the activity.

A repeated S10 fraction was subjected to electrophoresis at pH 8·5 in veronal buffer and then at pH 5·4 in sodium acetate–acetic acid buffer supported with NaCl. At pH 8·5 the schlieren diagram of this preparation showed two peaks (Fig. 4*a*). The ratio of the area under the peaks suggested that the symmetrical leading peak was 95% pure. When the same preparation was subjected to electrophoresis at pH 5·4 only one asymmetric peak developed (Fig. 4*b*). This evidence suggested that this preparation consisted of only two components, the predominant one being paraoxonase.

Preparative boundary electrophoresis. Attempts were then made to separate the leading components by taking advantage of the different migration velocities in veronal buffer, pH 8·5. The large preparative cell with one closed electrode of the Tiselius apparatus (Perkin–Elmer model) was employed. The ascending limb communicated with the closed electrode, which had a gassing device permitting the boundary to be displaced backwards.

Preparative electrophoresis was successful with run 31. The bottom and descending limbs of the cell were filled first with a 1% protein solution in 0·05 M-veronal buffer, pH 8·5. The electrophoresis was run at 130 V and 7 mA and the progress of the boundaries was observed at intervals. The relative positions of the two boundaries just before the leading boundary disappeared from the ascending limb (135 min.) is shown in Fig. 6. Electrophoresis was continued for a total of 195 min., when the leading boundary disappeared entirely from the ascending limb. The boundaries were then displaced back with a gassing apparatus until the rear boundary had disappeared into the bottom limb (24 min.). The limbs were then isolated.

Electrophoresis of the material obtained from the ascending limb showed one component (Fig. 7). A small distortion appeared at the rear shoulder of the boundary in the descending limb, but since it did not occur in the ascending boundary or change

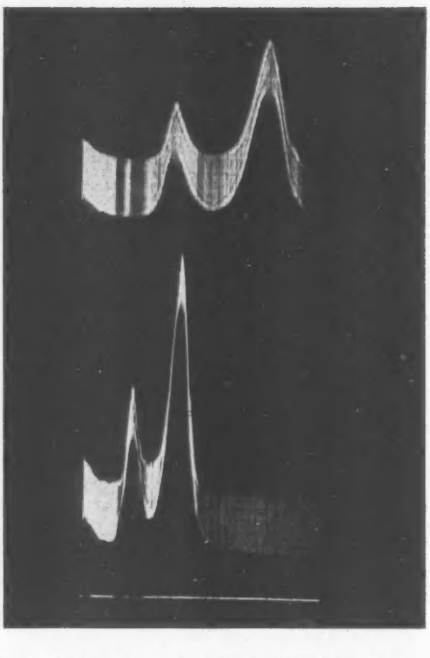


Fig. 5. Analytical boundary electrophoresis; the schlieren diagram of the S10 fraction from which part of the material represented by the rear boundary was separated and analysed. Descending limb; pH 8·2, 0·1 M-veronal buffer; 108 V, 5·5 mA; top, 120 min.; bottom, 58 min.

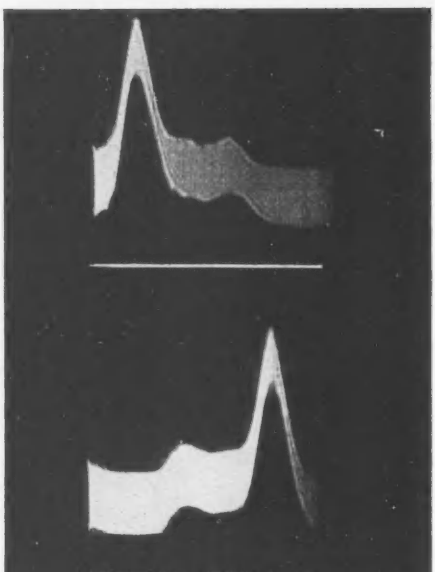


Fig. 6. Preparative boundary electrophoresis; the schlieren diagram of the S10 repeated fraction just before the main peak left the ascending limb. Top, ascending limb; bottom, descending limb.

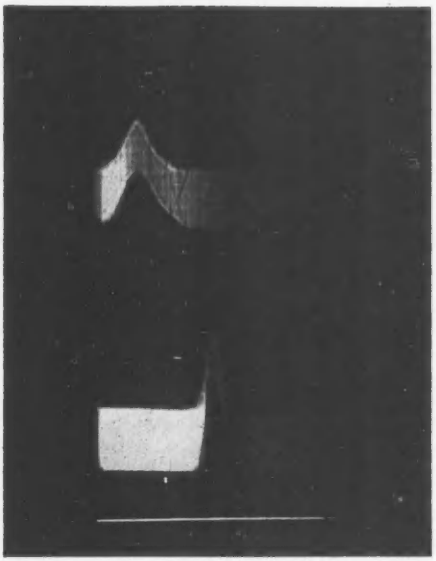


Fig. 7. Analytical boundary electrophoresis; the schlieren diagram of the component isolated by preparative electrophoresis. Ascending limb: top, 51 min.; bottom, 85 min.; pH 8·4; 0·05M-veronal buffer; 140v, 3·8 mA.

Table 4. *Specific activity of the material from run* 31 *in the limbs of the preparative electrophoresis cell*

The activities were determined after electrophoresis and boundary displacement. The specific activity is in terms of μmoles of *p*-nitrophenol liberated/mg. of protein/30 min. at $V_{max.}$. The paraoxonase activities are as percentages of the activity recovered finally.

Limb	4 mM-Paraoxon specific activity	Paraoxonase activity (%)
Ascending	4·76	36
Bottom	3·70	27
Descending	3·26	37

its position relative to the main peak during the electrophoresis, it is probable that it did not represent another component. It seemed justifiable to conclude that the material shown in Fig. 7 was electrophoretically pure paraoxonase with a mobility of $0{\cdot}88 \pm 0{\cdot}01 \times 10^{-4}$ cm.^{2}/v/sec.

Repeated electrophoresis resulted in a continuous loss of activity. In run 31 about 80 % of the activity was lost after the final electrophoresis. Part of the loss was caused by repeated electrophoresis due to gassing of the electrodes. Analysis

Table 5. *Paraoxon* K_m *of the fractions in run* 31

K_m was determined from colorimetric activity determinations, which were plotted according to the Dixon (1953) modification of the Lineweaver & Burk plot (1934).

Fraction	Purification	K_m (mM)
Serum	1	0·29
P2	84	0·38
P4	30	0·58
S5	56	1·0
S7	127	1·7
S10	310	4·4

of the contents of the three limbs confirmed that the activity was in the ascending limb (Table 4).

Paper electrophoresis. Various fractions from the purification procedure were examined by paper electrophoresis at pH 8·5 in 0·1M- and 0·05M-veronal buffer. The protein in fraction S9 resolved into three zones, with the activity in the leading zone. The papers were examined under ultraviolet light and the position of the fluorescence in the leading zone was marked. The papers were then dipped in paraoxon-buffered substrate and the position of the activity was marked by the appearance of yellow *p*-nitrophenol. The position of the protein was determined either with ninhydrin or Amido schwarz 10B dye (1-amino-8-hydroxy-3:6-disulphonic acid-2-diazo(*p*-nitrophenyl)-7-diazophenyl naphthalene). In every instance the position of the activity and fluorescence coincided with the protein in the leading zone. More purified fractions (S 10 etc.) tended to remain at the origin, but when the paper was overloaded a crescent-shaped zone moved down the paper. The coincidence of the fluorescence, activity and all the protein in these patterns was taken to confirm the essential homogeneity of these preparations.

The protein in the active zone tended to stain more lightly with Amido schwarz 10B dye and more brilliantly with ninhydrin than the protein in the other zones, suggesting a relatively small molecule.

Ultracentrifuging. On the basis of a single determination the sedimentation coefficient was 3·69s (uncorrected). This suggested a molecular weight of 35 000 to 50 000 according to Svedberg & Pedersen (1940). The schlieren diagram of the material used is shown in Fig. 4. The ultracentrifuge picture showed one symmetrical peak.

Increase in the paraoxon K_m. The K_m of successive fractions from the purification procedure progressively increased. The K_m doubled during the first 30-fold purification and increased seven times with the final ten-fold purification (Table 5).

The increased K_m suggested the presence of an activator in sheep serum which was lost during purification. Repeated dialysis of serum and other

fractions did not result in a loss of activity, suggesting that the activator, if present, was a relatively large molecule.

The K_m did not increase significantly beyond 4·4 mM in S10 despite further purification, suggesting that the activator had been removed by this stage (Table 6). Partial irreversible deactivation of an electrophoretically homogeneous sample (Fig. 4) did not alter the K_m.

If the increase in the K_m of retained fractions reflected the removal of an activator from the bulk of the enzyme, then the K_m of the discarded fractions containing the activator would be significantly lower than the K_m of either the retained fraction or the starting material. The K_m of the retained fraction S7 and the starting material P6 was 0·17 mM and 0·10 mM respectively, whereas that of the discarded fraction P7 was 0·72 mM. Similar results were found for the fractions of stage 5. These results also suggest that the activation was reversible.

Recovery of activity. The recovery of a stage was the sum of the activity in the precipitate and supernatant, expressed as the percentage of the activity in the starting material. Low recoveries were observed in the later stages of the purification procedure when the activity determinations were made with 4 mM-paraoxon. Recalculation of the recovery with the maximum velocity associated with the K_m of each fraction increased the recovery, suggesting that the apparent loss could be largely accounted for by the change in K_m (Table 7). The overall recovery to stage 10 increased from an apparent 1060 units to 2260. The calculated purification increased by a comparable ratio.

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2	S1	9 650	9 680	100	10 550	10 400	100
4	S3	9 000	8 120	95	9 480	9 280	98·5
5	P4	6 210	5 550	89	7 060	6 920	98
7	P6	4 300	3 140	73	4 970	4 450	90
10	P8	1 720	1 060	62	2 450	2 260	92

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The temperature stability of a S10 fraction, purified 250-fold, is shown in Fig. 8. Above 42° the activity was rapidly destroyed. At 37° the enzyme was stable for 3 hr. and for longer periods at room temperature.

When stored at 2° purified preparations lost activity at the rate of about 7 % per day at pH 7·6. When a purified preparation was frozen from a weak bicarbonate solution, 10 % of the activity was lost but the remainder of the activity was retained for at least 10 weeks. The paraoxonase activity of frozen serum remained intact over a period of months.

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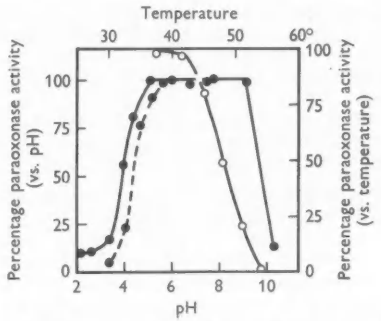
5	—	0·05	96
1	—	0·53	60
0·5	—	0·79	40
0·18	—	0·86	35
0·09	—	1·12	13
—	1·0	0·10	93
—	0·132	0·88	33
—	0·066	0·96	27
0·18	0·132	0·44	67
0·07	0·066	0·84	37
0·5	1·0	0·06	95



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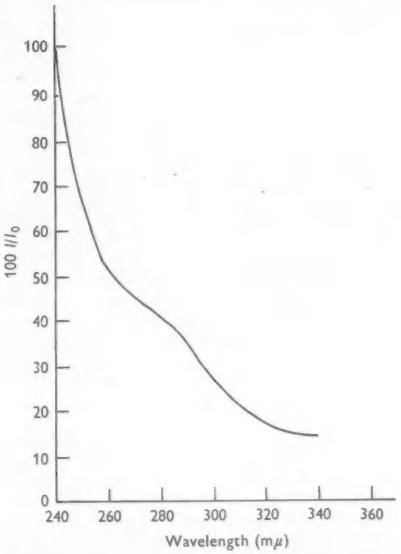

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fractions did not result in a loss of activity, suggesting that the activator, if present, was a relatively large molecule.

The K_m did not increase significantly beyond 4·4 mM in S10 despite further purification, suggesting that the activator had been removed by this stage (Table 6). Partial irreversible deactivation of an electrophoretically homogeneous sample (Fig. 4) did not alter the K_m.

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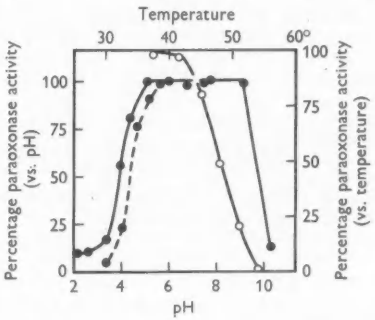

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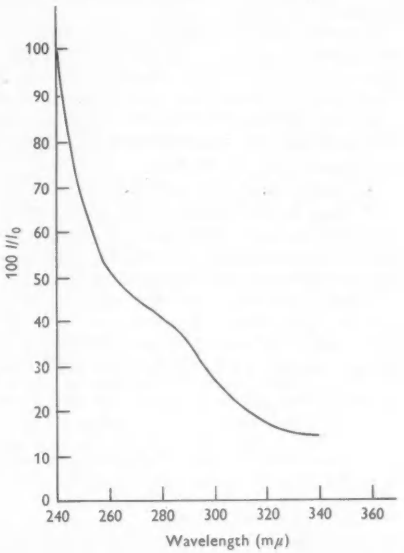

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ultraviolet region and that their absorption peaks overlap. The ratio of the absorptions at 260 mμ compared with that at 280 mμ was 0·85 for purified material. The ratio of the combined proteins of the serum was 1·63.

Phosphorus analysis indicated the presence of 1·5% of phosphorus in the paraoxonase, on the assumption that paraoxonase protein was 16% nitrogen. The phosphorus to nitrogen ratio of S10 fractions was about 10 times that of the average for serum protein. When a repeated S10 fraction was run chromatographically on silica-impregnated paper, according to the method of Lea, Rhodes & Stoll (1955), choline-containing spots were detected at the origin and near the solvent front parallel to lecithin. The reaction for choline was strong as judged by the method of Chargaff, Levine & Green (1948). These results suggested that paraoxonase contained phospholipid, part of which was loosely bound and came off readily in the chloroform solvent. The absence of streaking suggested that the choline-containing component remaining at the origin was tightly bound.

SUMMARY

1. The enzyme in sheep serum which hydrolyses diethyl *p*-nitrophenyl phosphate (paraoxon) has been purified 330- to 385-fold. Electrophoretic evidence suggests that the product was 80–95% pure and contained only one other, non-active, component.

2. Low-temperature ethanol, pH and ionic-strength fractionation resulted in a 114-fold purification with a 32% yield. Ammonium sulphate fractionation increased the purification to 300-fold and lowered the yield to about 15%. Repeated ammonium sulphate fractionation brought the purification to 330- to 385-fold.

3. The paraoxon K_m of purified fractions (4·2 mM) was 15 times that of sheep serum (0·29 mM). The characteristics of the increase in K_m suggests the presence of a paraoxonase activator in sheep serum which was removed during purification.

4. Paraoxonase solutions fluoresced blue when exposed to ultraviolet radiation.

5. Analysis of the enzyme indicated the presence of about 1·5% of phosphorus and chromatographic experiments indicated the presence of choline-containing phospholipids.

6. The sedimentation constant and other observations suggest a molecular weight of 35 000–50 000.

I am particularly grateful to Dr E. C. Webb for his generous help and advice on the presentation of this paper. I would also like to thank Dr M. Naughton for his help in running the chromatograms for phospholipid and Dr M. Dixon for running the spectrum shown in Fig. 9 on the Beckman D-K recording spectrophotometer. Albright and Wilson Ltd. have provided a generous supply of paraoxon, for which they have my sincere thanks.

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Mitochondria of the Ehrlich Ascites-Tumour Cell

ISOLATION AND STUDIES OF OXIDATIVE PHOSPHORYLATION

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Isolation of mitochondria from the Ehrlich ascites tumour is complicated by the fact that these cells have an extremely tough membrane which is not easily ruptured. The tumour cells can be disrupted by the application and sudden release of gas pressure (Nirenberg & Hogg, 1956), or by grinding in sucrose solution with all-glass or Teflon Potter homogenizers (Kun, Talalay & Williams-Ashman, 1951; Mutolo & Abrignani, 1958), or by vigorous shaking with quartz sand (Lindberg, Ljunggren, Ernster & Rēvēsz, 1953). These methods are, however, tedious and do not result in complete homogenization. Cytoplasmic material with included mitochondria remains attached to the nuclei in such sucrose homogenates, and hence a certain amount of the mitochondrial fraction is lost during initial centrifuging to remove the nuclei. Sonic oscillation has been applied to disrupt Ehrlich ascites cells (Davidson, Smellie, Keir & McArdle, 1958), but, since this technique may result in damage to the mitochondria, homogenization methods are to be preferred.

Unpublished work by E. Stotz and co-workers has shown that the membrane of ascites cells can be easily ruptured with a Dounce homogenizer (Dounce, Witter, Monty, Pate & Cottone, 1955) if these cells are previously swollen for 30–60 min. in diluted (1:3) Ringer–phosphate solution. The technique of lysing the cells into water has been applied by Littlefield & Keller (1957) to isolation of the microsome fraction from Ehrlich ascites-tumour cells.

In our experiments the cell membranes were prestressed in hypotonic solution and the cells subsequently disrupted into pure water by osmotic shock. Immediately after lysis, sufficient concentrated sucrose solution was added to the homogenate to give the usual 0·25 M-sucrose suspension, from which mitochondria were isolated in the customary manner. The method results in clean nuclei and mitochondria free of cytoplasmic material.

Unlike liver mitochondria, those of the Ehrlich ascites cells do not appear to be damaged by contact over short periods with hypotonic solutions. With our method we have obtained preparations which show oxidative-phosphorylation activity with succinate, α-oxoglutarate and glutamate as substrates.

MATERIALS AND METHODS

Ehrlich ascites tumour. This tumour was kindly supplied by Dr K. Sugiura of the Sloan-Kettering Institute, New York, and has been maintained in Swiss albino mice which were used between the ninth and twelfth day after inoculation.

Hexokinase. Crystalline material was supplied by General Biochemicals Inc., Ohio, U.S.A. The enzyme was assayed by measurement of hexose disappearance according to Somogyi (1952), and by measurement of the disappearance of acid-labile phosphorus according to Crane & Sols (1955). Preparations were found to have an activity of approximately 150 units/mg.

Cytochrome c. A 1% (w/v) solution in aq. 0·09% NaCl was supplied by General Biochemicals Inc., and found to contain $5{\cdot}87 \times 10^{-7}$ moles/ml. when assayed according to Umbreit, Burris & Stauffer (1957).

Adenosine 5′-phosphate and adenosine diphosphate. These were used as supplied by General Biochemicals Inc., and by the H.M. Chemical Co. of Los Angeles, California, respectively.

Adenosine triphosphate. This was used as the tetrasodium salt supplied by General Biochemicals Inc., and assayed 85% pure by the method of Crane & Sols (1955).

Diphosphopyridine nucleotide. This was obtained from General Biochemicals Inc., and was assayed 85% pure by a modification of the method of Brodie (1955) using 10 mg. of sodium dithionite/1·0 ml. of 0·2 M-disodium hydrogen phosphate as the reducing solution.

All other substrates and chemicals used were of analytical reagent grade.

Isolation of mitochondria. All operations were carried out at 0–4°. Mice were killed by cervical fracture and ascites fluid was withdrawn from the peritoneal cavity with a sterile syringe. The fluid was mixed immediately with sodium citrate solution [1 ml. of 3·8% (w/v) sodium citrate/10·0 ml.] and stored in ice. The cells were collected by centrifuging at 1800 *g* for 10 min. and washed once or twice by resuspending in iso-osmotic NaCl solution. They were then dispersed in hypo-osmotic (¼ strength) Ringer–phosphate solution and allowed to stand 15–20 min. in the cold with occasional stirring. Phase-contrast microscopy at

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this stage showed the ascites cells to be swollen but not lysed. Contaminating erythrocytes were completely lysed. The suspension was then centrifuged at 1800 *g* for 10 min. and the supernatant discarded. The cells (6–8 g. wet wt.) were immediately resuspended in ice-cold water (60 ml.) for exactly 3·0 min. by using a Dounce homogenizer with loose plunger (clearance approx. 0·001 in.) as supplied by Blaessig Glass Specialities, Rochester, N.Y., U.S.A. [To ensure complete lysis and removal of adhering cytoplasm from the nuclei, it is important to use 8–10 vol. of water/g. (wet wt.) of cells.] Concentrated sucrose solution was then quickly added to give the usual 0·25 M-sucrose suspension, which was further homogenized with 6–8 passes of the loose plunger, followed by 15 passes with the tight plunger (clearance approx. 0·0005 in.). Phase-contrast microscopy at this stage revealed a 90–95% homogenate in which the nuclei were free of cytoplasmic material. The pH of the homogenate measured at 25° with the glass electrode was 7·1.

Nuclei and cell debris were removed by centrifuging at 600–700 *g* for 20 min. The supernatant was carefully withdrawn and centrifuged 10 min. at 12 000–13 000 *g* in the high-speed head of the M.S.E. Major Refrigerated Centrifuge. The sediment from this centrifuging consisted of a firmly packed mitochondrial pellet with a so-called 'fluffy' layer loosely packed above it. The mitochondrial pellet was resuspended in 0·25 M-sucrose by using the homogenizer and again centrifuged for 10 min. at 12 000 *g*. Two such washings effectively removed the 'fluffy' layer and gave a uniform mitochondrial pellet which was finally resuspended in 0·25 M-sucrose [approx. 5–6 ml./g. (wet wt.)] equivalent to a concentration of 3–11 mg. of mitochondrial protein/ml. The pH of the final suspension measured with the glass electrode was 6·9. (In certain cases the mitochondrial suspension after the first washing was recentrifuged at 600–700 *g* for 10 min. to ensure complete removal of nuclei.)

Mitochondrial protein content. This was determined according to the biuret method of Cleland & Slater (1953) with an ovalbumin standard.

Measurement of oxidative phosphorylation. The following medium in a volume of 2·36 ml. was placed in the main compartment of each Warburg flask (molarities refer to the final volume of 3·0 ml. after addition of the side-arm contents and mitochondrial suspension): 0·045 M-sodium potassium phosphate buffer, pH 7·4; 15 mM-KF; 8 mM-$MgCl_2$; 25 mM-adenosine diphosphate (ADP); mM-adenosine 5′-phosphate (AMP); 2 mM-ethylenediaminetetraacetic acid (EDTA); 34 mM-D-glucose; 0·01 M-KCl; 62 mM-sucrose; 0·24 mM-diphosphopyridine nucleotide (DPN); 7 mM-substrate; 9·8 μM-cytochrome *c*. (In measurement of α-oxoglutarate oxidation 0·02 M-malonate was added as an inhibitor. AMP, ADP and all substrates were neutralized to pH 7·4 with KOH and stored at −30° before use.) The side arms of each flask contained 70% of the total D-glucose, together with 1–3 mg. of crystalline hexokinase, in a volume of 0·24 ml.

To each Warburg vessel was added 0·4 ml. of a mitochondrial suspension in 0·25 M sucrose, giving a concentration of 0·4–1·5 mg. of protein/ml. of incubation medium after addition of the side-arm contents. The centre well of each flask contained 0·2 ml. of 40% (w/v) KOH for CO_2 absorption, and the gas phase was air. Oxygen uptake was measured for 25–30 min. after 13 min. equilibration at 30°. Phosphate uptake was determined by the method of Fiske & Subbarow (1925), on cold trichloroacetic acid filtrates. The oxygen uptake of the mitochondria (q_{O_2}) was expressed as μl. of O_2/mg. of protein/hr. according to Slater & Holton (1954).

RESULTS

The results in Table 1 indicate that once-washed mitochondria have a low protein content and show low P:O ratios with succinate and α-oxoglutarate as substrates. The oxygen uptake per mg. of protein of once-washed mitochondria is, however, comparable with that of twice-washed mitochondria and is even higher when succinate is used as substrate. The yield of once-washed mitochondria corresponds to 6–8% of the wet weight of whole cells, while the yield of twice-washed material is equivalent to 4–6%. A second washing results in a uniform mitochondrial pellet free of 'fluffy' layer. Twice-washed mitochondria were thus used for the experiments recorded in Tables 2 and 3. A force of 12 000 *g* was found optimum for

Table 1. *Effect of washing on yield and oxidative phosphorylation of Ehrlich ascites-cell mitochondria*

Each flask contained the following in a total volume of 3·0 ml.: mitochondrial suspension in 0·25 M-sucrose, 0·4 ml.; sodium potassium phosphate buffer (pH 7·4), 45 mM; D-glucose, 34 mM; KF, 15 mM; $MgCl_2$, 8 mM; ADP, 2·5 mM; AMP, mM; EDTA (pH 7·4), 2 mM; KCl, 0·01 M; sucrose, 67 mM (with succinate as substrate), or 62 mM (with α-oxoglutarate as substrate); succinate, 0·01 M, or α-oxoglutarate, 6·6 mM; cytochrome *c*, 9·8 μM; 1–2 mg. of crystalline hexokinase. (In experiments with α-oxoglutarate as substrate, malonate (0·02 M), DPN (2·4 mM) and nicotinamide (0·02 M) were added in addition.) Temp. 30°. Equilibration time 13 min. Manometric flasks were maintained at 0° before each experiment.

	Mitochondria					
			Succinate		α-Oxoglutarate	
No. of washings	Yield [% (wet wt.) of whole cells]	Protein content [mg./g. (wet wt.)]	P:O	q_{O_2}	P:O	q_{O_2}
1	6–8	14·6	0·26	146·0	0·82	46·8
2	4–6	26·0	1·44 ± 0·20*	105·9 ± 22·2*	2·38†	53·8†

* Average of thirteen different mitochondrial preparations.
† Average of two different mitochondrial preparations.

obtaining a firmly packed mitochondrial pellet with high protein content. The final mitochondrial suspension in 0·25 M-sucrose was apparently homogeneous when examined under the phase-contrast microscope.

Mitochondrial preparations showed q_{O_2} values in the range 77–149 with succinate as substrate (Table 2). A final concentration of 0·24 mM-DPN was present in each flask, although this is probably not required for the oxidation of succinate. The P:O ratios with succinate as substrate were measured for 15 of the mitochondrial preparations by using an active hexokinase and were found to lie within the range 1·01–1·82. Six of the preparations showed P:O ratios in the range 1·54–1·82.

With ascorbate, mitochondria showed q_{O_2} values in the range 54–66, but it proved impossible to measure P:O ratios with this substrate. Inorganic phosphate was liberated, instead of esterified, possibly owing to the operation of the myokinase reaction combined with a considerable degree of uncoupling at the cytochrome *c* level of oxidative phosphorylation. Similar results were obtained with or without the addition of 0·24 mM-DPN. In each experiment with ascorbate, a control determination was carried out on the same batch of mitochondria with succinate as substrate. This was not only to ensure the functional integrity of the mitochondrial preparation, but to provide additional information regarding the phosphorylation site(s) which appear to be uncoupled in tumour-cell mitochondria.

In experiments with α-oxoglutarate as substrate (Table 3), mitochondria showed q_{O_2} values of approximately 30 in media unfortified with DPN. Addition of 0·24 mM-DPN had little effect on the q_{O_2}, but 2·4 mM-DPN (with 0·02 M-nicotinamide) raised the q_{O_2} by approximately 100%, with a concomitant increase in P:O ratio. It is noteworthy that 0·02 M-nicotinamide alone raised the P:O ratio, but was apparently without effect on the q_{O_2} in any experiment.

A similar DPN effect was observed with L-glutamate as substrate (Table 3). Addition of 0·24 mM-DPN to the medium raised the q_{O_2} by 115%, while a 400% increase was observed on addition of 1·2 mM-DPN with 0·02 M-nicotinamide. The P:O ratio was also increased by addition of 1·2 mM-DPN with 0·02 M-nicotinamide, but the enhancement was less than was observed on similar fortification of the medium with α-oxoglutarate as substrate.

Fig. 1 shows the change in oxygen uptake per mg. of protein of various mitochondrial preparations with four different substrates over experimental periods of 30–35 min. Curves are included to show the effect of DPN and nicotinamide additions in the oxidation of α-oxoglutarate and L-glutamate.

Table 2. *Oxidation and associated phosphorylations with succinate and ascorbate respectively as substrates*

Each flask contained the following in a total volume of 3·0 ml.: mitochondrial suspension in 0·25 M-sucrose, 0·4 ml.; sodium potassium phosphate buffer (pH 7·4), 45 mM; D-glucose, 34 mM; KF, 15 mM; $MgCl_2$, 8 mM; ADP, 2·5 mM; AMP, mM; EDTA (pH 7·4), 2 mM; KCl, 0·01 M; sucrose, 67 mM (in experiments without DPN), or 62 mM (in experiments with DPN); succinate, 0·01 M, or ascorbate, 6·6 mM; cytochrome *c*, 9·8 μM; 1–2 mg. of crystalline hexokinase. Temp. 30°. Equilibration time 12–15 min. Manometric flasks were maintained at 0° before each experiment.

Expt.	DPN additions (m-moles)	Incubation time (min.)	Wt. of protein/flask (mg.)	ΔO (μg. atom)	ΔP_i (μmole)	P:O	q_{O_2}	Remarks
Succinate								
1	0·24	32	2·17	−7·36	−4·95	0·87	78·8	P:O ratios low owing to use of hexokinase with low activity
	0·24	32	2·17	−7·16	−4·78	0·67	76·9	
	0·24 (No KF added)	32	2·17	−9·26	−7·73	0·84	99·6	
2	None	33	2·29	−6·64	−6·71	1·01	65·6	—
3	0·24	32±4	2·64±0·78	−(10·97±1·63)	−(15·82±3·71)	1·44±0·21	105·9±22·2*	Average of 13 different mitochondrial preparations
4	0·24	35	2·83	−11·15	−13·03	1·17	83·9	—
Ascorbate								
1	None	33	2·29	−6·70	+0·93	—	66·2	Same mitochondrial preparation as used for Expt. 2 with succinate as substrate
2	None	33	2·29	−6·58	+4·66	—	65·0	
3	0·24	35	2·83	−7·14	+2·81	—	53·8	Same mitochondrial preparation as used for Expt. 4 with succinate as substrate

* Average deviation from mean.

Table 3. *Oxidation and associated phosphorylations with α-oxoglutarate and L-glutamate respectively as substrates*

Each flask contained the following in a total volume of 3·0 ml.: mitochondrial suspension in 0·25 M-sucrose, 0·4 ml.; sodium potassium phosphate buffer (pH 7·4), 45 mM; D-glucose, 34 mM; KF, 15 mM; $MgCl_2$, 8 mM; ADP, 2·5 mM; AMP, mM; EDTA (pH 7·4), 2 mM; KCl, 0·01 M; sucrose, 62 mM; α-oxoglutarate, 6·6 mM, or L-glutamate, 6·6 M; malonate, 0·02 M; cytochrome *c*, 9·8 μM; 1–2 mg. of crystalline hexokinase. (In experiments with L-glutamate as substrate, malonate was omitted from the incubation medium.) Temp. 30°. Equilibration time 13 min. Manometric flasks were maintained at 0° before each experiment.

Expt.	Special additions	Incubation time (min.)	Wt. of protein/flask (mg.)	ΔO (μg. atom)	ΔP_i (μmole)	P:O	q_{O_2}
α-Oxoglutarate							
1	—	35	1·98	−3·17	−5·87	1·85	34·1
2	0·24 mM-DPN	32	4·35	−5·80	−11·28	1·95	31·1
3	2·4 mM-DPN + 0·02 M-nicotinamide	35	1·98	−5·41	−12·97	2·40	58·3
4	0·02 M-Nicotinamide	35	2·12	−2·61	−6·43	2·47	26·2
	2·4 mM-DPN + 0·02 M-nicotinamide	35	2·12	−4·92	−11·59	2·36	49·4
L-Glutamate							
1	1·2 mM-DPN + 0·02 M-nicotinamide	30	2·46	−6·03	−10·97	1·83	60·9
	—	30	2·46	−1·48	−1·48	1·00	15·0
2	0·24 mM-DPN	30	1·56	−2·36	—	—	37·6

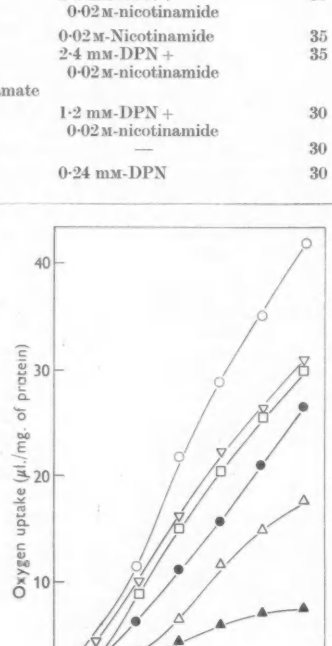

Fig. 1. Oxygen uptake of mitochondria in the presence of various substrates and cofactors. Incubation medium as in Tables 1–3 with 0·01 M-succinate (○), 6·6 mM-L-glutamate + 1·2 mM-DPN + 0·02 M-nicotinamide (▽), 6·6 mM-α-oxoglutarate + 2·4 mM-DPN + 0·02 M-nicotinamide (□), 6·6 mM-ascorbate (●), 6·6 mM-α-oxoglutarate (△), 6·6 mM-L-glutamate (▲). Temp. 30°.

DISCUSSION

If carefully followed, the method of isolation of ascites-tumour mitochondria described offers several advantages over previous techniques (Nirenberg & Hogg, 1956; Kun *et al.* 1951; Mutolo & Abrignani, 1958, and Lindberg *et al.* 1953). The use of Dounce homogenizers, which need not be removed from the ice bath during the isolation procedure, is convenient for proper control of temperature. The homogenization of pre-stressed cells is relatively simple in comparison with the prolonged grinding of unswollen cells required in previous isolation techniques.

The mitochondria obtained showed no tendency to agglutinate in water or 0·25 M-sucrose and appeared to be functionally intact, as evidenced by the oxidative phosphorylation activity found with a variety of substrates. Unlike those of liver, the particulates showed no endogenous oxygen uptake. A yield of 4–6% of the original wet weight of cells as twice-washed mitochondria is considered satisfactory on account of the low mitochondrial content of tumour cells (Kit & Griffin, 1958).

In view of the finding by Fonnesu & Davies (1956) that AMP prevents swelling of liver mitochondria, certain experiments were carried out in which the Ehrlich cells were lysed into water containing mM-AMP. Mitochondria obtained by this modification did not, however, show any difference in oxidative phosphorylation capacity from others isolated without addition of AMP.

The fact that once-washed mitochondria showed low P:O ratios with succinate and α-oxoglutarate (Table 1), combined with the observation that twice-washed mitochondria showed a lower q_{O_2} with succinate than once-washed mitochondria (Table 1), would indicate the presence of either

contaminating ATP-ases or uncoupling agents in once-washed mitochondrial preparations. Twice-washed mitochondria were accordingly used for all subsequent experiments in Tables 2 and 3.

In a medium based on that of other workers, the oxidation of succinate by Ehrlich-tumour mitochondria was accompanied by a marked esterification of inorganic phosphate (cf. Kun *et al.* 1951; Lindberg *et al.* 1953). P:O ratios determined with succinate lay within the range 1·01–1·82. The variation may be due to slight differences in age or the amount of contaminating erythrocytes, leucocytes, etc., in each batch of ascites fluid used. Minor variations of experimental technique during the isolation procedure may also account for the range of results. The P:O ratios compare favourably with the values of 1·4, obtained by Lindberg *et al.* (1953), and 1·2, obtained by Kun *et al.* (1951), for Ehrlich-ascites mitochondria.

The theoretical P:O ratio of 2·0 for succinate was never obtained, even in the highly fortified medium of Table 2. The presence of fluoride decreased the q_{O_2} of one batch of mitochondria from 99 to 78 in accord with the hypothesis of Slater (1954) that formation of a phosphate–fluoride complex inhibits succinic dehydrogenase in mitochondria. Addition of 0·24 mM-DPN to the phosphorylating medium probably served to maintain structural integrity of the mitochondria but, as might be expected, it was without effect on the q_{O_2} and P:O ratios with succinate as substrate. Ehrlich ascites-cell mitochondria showed q_{O_2} values with succinate which, per mg. of protein, were comparable with those of kidney, but which were in general above those of other normal cells, e.g. liver and brain (Wenner & Weinhouse, 1953).

Given ascorbate as substrate, Ehrlich ascites-cell mitochondria showed q_{O_2} values of 54–66 in phosphorylating media, with or without addition of DPN. In one case oxygen uptake was equivalent to that of the succinate control which was run on the same batch of mitochondria. In no instance could a P:O ratio be determined with ascorbate as substrate. Instead of phosphate esterification a net increase of inorganic phosphate was consistently observed. Even the high concentration of fluoride present in the medium was apparently without effect in preventing adenosine triphosphatase activity at the phosphorylation level concerned in ascorbate oxidation (cf. Emmelot & Bos, 1957).

Failure to achieve the theoretical P:O ratio of 2·0 during succinate oxidation would indicate either that the two phosphorylating sites between succinic dehydrogenase and cytochrome a_3 (oxidase) are both partially uncoupled, or that one of the sites is uncoupled to a considerable extent. The experiments with ascorbate in which no phosphate esterification could be found would suggest that it is the cytochrome *c* phosphorylation site that is very largely uncoupled in Ehrlich ascites-cell mitochondria (cf. Copenhaver & Lardy, 1952; Slater, 1955; Hunter, 1951; Nielsen & Lehninger, 1954 and Chance & Williams, 1956). This may, of course, be true only for mitochondria prepared by the method described in this paper. Uncoupling of the cytochrome *c* phosphorylation site has not been observed in normal cell mitochondria such as those of liver (cf. Nielsen & Lehninger, 1954).

Studies with substrates that require DPN for their oxidation revealed interesting data on the functioning of Ehrlich ascites-cell mitochondria. Thus, in the absence of added DPN or the diphosphopyridine nucleotidase inhibitor nicotinamide, mitochondria oxidized α-oxoglutarate with low q_{O_2} values and P:O ratios which were little affected by addition of 0·24 mM-DPN to the medium. However, in experiments where the concentration of DPN was raised to 2·4 mM and nicotinamide was also added, a marked stimulation in both q_{O_2} and P:O ratio was observed. The highest P:O ratio for α-oxoglutarate oxidation (P:O 2·7) was obtained in the presence of nicotinamide alone without DPN addition.

Low oxygen uptakes and P:O ratios were obtained with L-glutamate as substrate. Addition of 0·24 mM-DPN to the medium caused a marked increase in q_{O_2}, but an even more striking increase in both q_{O_2} and P:O ratio was observed on addition of 1·2 mM-DPN together with nicotinamide.

These results clearly confirm the DPN requirement of tumour mitochondria discovered by Wenner & Weinhouse (1953), i.e. the Wenner–Weinhouse effect. These workers found that oxidation of pyruvate and citric acid-cycle metabolites by tumour mitochondria was greatly enhanced by the addition of DPN. The effect was, moreover, specific for DPN and could not be simulated by adding any other known cofactor. Wenner & Weinhouse suggested that tumour mitochondria may be less efficient in binding DPN than are their counterparts in non-neoplastic tissues, and that this may result in a correspondingly high level of DPN in the cytoplasm of the tumour cell. The finding of Branster & Morton (1956), that the rate of DPN synthesis in isolated tumour nuclei was approximately one-fifth of that in corresponding normal cell nuclei, would suggest that tumour mitochondria are unable to obtain an adequate supply of the coenzyme for proper biological functioning in the living cell, although the lower nuclear DPN synthesis may even be adequate for the limited number of mitochondria in tumour cells. The possibility also exists that DPN transport between the nucleus and mitochondria may be impaired in tumour cells.

Our observation that Ehrlich ascites-cell mitochondria show a high P:O ratio for α-oxoglutarate oxidation without added DPN, but in the presence of the nicotinamide alone, would suggest that these tumour mitochondria may indeed have sufficient bound DPN for α-oxoglutarate oxidation, but will function satisfactorily only if the bound DPN is not broken down by diphosphopyridine nucleotidase action. A high diphosphopyridine nucleotidase activity in tumour-cell mitochondria has been observed by many workers (Morton, 1958; Kun *et al.* 1951; Weinhouse, 1955).

SUMMARY

1. An improved method is described for the isolation of Ehrlich-tumour mitochondria. The ascites cells are pre-stressed by swelling in hypotonic solution and are then disrupted into pure water by osmotic shock. Addition of concentrated sucrose solution to the homogenate immediately after lysis gives a 0·25 M-sucrose suspension, from which mitochondria can be isolated by differential centrifuging in the customary manner.

2. The method affords clean mitochondria which are free of adhering cytoplasmic material and are functionally intact, as evidenced by their oxidative phosphorylation activity shown with succinate, α-oxoglutarate and L-glutamate as substrates.

3. Studies with succinate and ascorbate as substrates have provided evidence which suggests that the cytochrome *c* phosphorylation site is uncoupled to a considerable extent in Ehrlich ascites-cell mitochondria.

4. Ehrlich ascites-cell mitochondria exhibit a pronounced diphosphopyridine nucleotide requirement for maximal oxidative phosphorylation activity with α-oxoglutarate and L-glutamate as substrates (Wenner–Weinhouse effect). Evidence suggests that the diphosphopyridine nucleotidase inhibitor, nicotinamide, may possibly be more effective than diphosphopyridine nucleotide itself in raising the P:O ratio.

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The Acid Mucopolysaccharides of the Sexual Skin of Apes and Monkeys

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The process of swelling of the sexual skin of immature female rhesus monkeys (*Macaca mulatta*) induced by oestrogenic stimulation, or the process occurring in mature pig-tail monkeys (*Macaca nemestrina*) and baboons (*Papio papio*), has been shown to be associated with a general water retention (Krohn & Zuckerman, 1937), an increase in the size of the cells of the connective tissue of the sexual skin (Aykroyd & Zuckerman, 1938) and with the presence of a viscous exudate which on occasions is easily drained from cut surfaces of the tissue. The exudate has a variable but high osmotic pressure (which may exceed that of serum) and a total protein content of about 2·5% (Fisher & Zuckerman, 1937).

Ogston, Philpot & Zuckerman (1939) obtained a substance, termed mucoprotein, which was precipitated from the exudate in 1% acetic acid and which contained 2–3% of hexosamine and 12–13% of nitrogen. This material was apparently responsible for the viscous nature of the exudate. Chain & Duthie (1940) observed that extracts of testes containing hyaluronidase decreased the viscosity of the exudate. Duran-Reynals, Bunting & van Wagenen (1950) showed that the roll swellings on the sexual skin of the rhesus monkeys could be collapsed by slight pressure after the swellings had been infiltrated with testicular hyaluronidase. These authors showed that the extracellular interfibrillar region in swollen sexual skin contained a metachromatically staining material sensitive to the hyaluronidases. These observations led to the conclusions that the exudate of swollen sexual skin contained hyaluronic acid and that the production of hyaluronic acid in the sexual skin was in some way concerned with the retention of water in this tissue.

Hyaluronic acid has been known for some time as a component of the amorphous ground substance of connective tissue and has, in company with chondroitin sulphate, been isolated from skin (Meyer & Chaffee, 1941; Pearce & Watson, 1949). There are some indications that hyaluronic acid is in some way connected with water retention in tissues. Pearce & Watson (1949) showed that in pretibial myxoedema the hyaluronic acid and chondroitin sulphate content of skin of the affected part increased several times over that of unaffected skin, and that the water content of affected skin was higher than that of unaffected skin. Hyaluronic acid accompanied by serum protein occurs in synovial fluid. Oedema of the joints occurs in some arthritics and is accompanied by the increased production of hyaluronic acid, apparently in depolymerized form (Ragan & Meyer, 1949). The concentration of hyaluronic acid in the fluid from the oedematous joints was not greater than that from normal joints and there was a marked rise in serum protein in the fluid from oedematous joints. Ropes, Bennett & Bauer (1939) obtained a much higher colloid osmotic pressure from normal synovial fluids than was calculated from their albumin and globulin content. The increased osmotic pressure was considered to be due to the 'mucin' (hyaluronic acid) but this is difficult to understand as the molecular weight of synovial fluid is estimated to be in the region of 10^7 (Ogston & Stanier, 1952).

Vitreous humour, which contains hyaluronic acid together with small amounts of protein as a gel, has been injected *in vivo* with hyaluronidase in an effort to remove hyaluronic acid (Pirie, 1949). The hyaluronic acid was 'disaggregated' but not removed from the ocular cavity. There was no diminution in vitreous volume, although the intraocular tension decreased considerably. The intraocular tension was regained in 9 days whereas the hyaluronic acid did not return to its aggregate state for 6 weeks.

Ludwig, Boas & Soffer (1950) induced proptosis in guinea pigs and noted that there was an increased water and hexosamine content of the loose connective tissue of the orbit. K. Aterman (personal communication, 1952) reversed proptosis by injecting hyaluronidase into the orbit.

These examples suggest that increased concentration of hyaluronic acid may bring about the retention of fluid in a tissue. Depolymerization leads to a decreased water retention. The oedema of arthritic joints, however, could be due to the marked accumulation of serum protein in the joint rather than an effect related to altered hyaluronic acid status. It is unsatisfactory attempting to draw general conclusions since the histological

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structures of the tissues mentioned are quite different, and this influences the possible mode of entry of water into them. While it was clear that hyaluronic acid was involved in the sexual-skin phenomenon it was by no means clear whether during the turgescent phase there was an increase in hyaluronic acid concentration and whether during deturgescence there was a change in polymerization or concentration of the hyaluronic acid or both. The sexual-skin phenomenon has now been investigated to gain information on these matters. The exact nature of the material in the exudate was investigated, since apart from the determination of hexosamine (Ogston *et al.* 1939) no chemical studies have been reported. Viscosity studies were carried out to determine whether there was any depolymerization of hyaluronic acid of the exudate in any phase of the sexual-skin cycle. Measurements were also made of the changes in concentration of hyaluronic acid and chondroitin sulphate occurring in the skin in the various phases of the cycle.

MATERIALS AND METHODS

Animals. Three species of animals were used: rhesus monkeys (*Macaca mulatta*), baboons (*Papio papio*) and pig-tail monkeys (*Macaca nemestrina*). With two exceptions, the specimens obtained from the baboons were from swellings occurring in normal menstrual cycles. The baboons were used in other experiments which caused the death of all but one of them. The pig-tail monkeys, which were obtained to replace the baboons with animals of convenient size and ones with a regular and consistent sexual-skin cycle, were found on arrival from Singapore to be sexually immature. One of these animals, on one occasion only, exhibited a naturally occurring sexual-skin swelling, and it was necessary to induce sexual-skin swellings by injection of oestrogens. With two exceptions, the swellings of the rhesus monkeys were brought about by oestrogen administration.

The animals were housed in cages in a room kept at 70° F. The staple foods were bread and steamed potatoes, supplemented when available by root vegetables, cabbages, apples and oranges. Vitamin supplements were given. Water was always available in the cages.

Specimens. Each animal was numbered and the occasions on which specimens were obtained were denoted by a letter. Thus 616A and 616B denote two occasions on which specimens of tissue were obtained from animal no. 616.

Sexual-skin swellings were induced experimentally with oestrone acetate, oestradiol-3-17β dipropionate or ethinyloestradiol dissolved in arachis oil. The injections were given intramuscularly in the legs. There is no apparent reason for regarding the swellings induced by one oestrogen as different from the swelling induced by another. Tables 1–3 list the relevant details pertaining to all the specimens from the three species used in this study.

Appearance of the sexual skins. Adolescent, pubertal and almost mature sexual-skin responses were obtained in rhesus monkeys (adequately described by Zuckerman, van Wagenen & Gardner, 1938). The scrotal and pubic-lobe swellings were observed to be soft but not pitting, whereas the roll swellings of the thigh and base of tail were very firm.

The baboons exhibited consistent sexual-skin changes, in which the swelling commenced during the latter part of, or immediately after, menstruation, rose to a maximum in a week and, after a further week in the fully swollen state, collapsed rapidly (within a few days). The baboon sexual-skin swelling was red and shiny and very resilient to pressure. The experimentally induced swellings in 616 did not appear to differ from the natural swellings except that the surrounding 'bare' area was much redder in the experimental swellings.

The sexual-skin swellings induced in the pig-tail monkeys were usually pale, dull and very hard. It was possible to pit them with considerable pressure but, on one occasion, at autopsy considerable haemorrhage was seen to have developed at the site of pressure. The shape of the sexual-skin swellings in the females of these animals varied, generally consisting of pubic-lobe swellings and a circum-anal swelling which extended to the base of the tail.

Table 1. *Details of baboons (female) used in experiments*

ODP, Oestradiol-3-17β dipropionate.

Specimen no.	No. of days since commencement of sexual-skin swelling	No. of days since commencement of collapse of sexual skin	State of sexual skin
615B	3	—	Early swelling
615D	10	1	Commencing to collapse
616A	8	—	Fully swollen
616C	3	—	Rapidly increasing
616D	16	3	Flabby and almost collapsed
616E	11	0	Fully swollen
616F	8	0	Fully swollen
616G	1	—	Very early swelling
616H	6	0	Fully swollen
616J	10	3	Collapsing
(Bilateral ovariectomy was carried out between 616J and 616K)			
616K 300 μg. of ODP/3 days (900 μg. total dose)		2	Large swelling commencing to collapse
616L 400 μg. of ODP/3 days (1200 μg. total dose)		—	Large swelling still increasing in size
621A	7	—	Fully swollen
624A	15	1	Collapsing, still large

Table 2. *Details of rhesus monkeys used in experiments*

OA, Oestrone acetate; ODP, oestradiol-3-17β dipropionate; EO, ethinyloestradiol.

Specimen no.	Sex	Hormone treatment (doses in μg.) (*a*) Routine	(*b*) Total dose of hormone	No. of days since last injection	State of sexual skin
575A	M.	500 OA/day	9000	1	Swollen scrotum. Other regions were pink
584A	F.	500 OA/day	7500	0	Swollen pubic lobe and peripheral quilting
609A	M.	500 OA/day	9000	1	Enormous scrotal swelling
655A	F.	Natural pubertal swellings consisting of large pubic lobes and peripheral rolls on the legs			
673A	F.	100 ODP/day + 100 mg. of cortisone/day	2100 500 mg.	1	Large swollen pubic lobe
673B	F.	—	—	—	Entirely collapsed
675A	M.	100 ODP/day	1400	0	Considerable scrotal swelling
675B	M.	100 ODP/day	1600	7	Swelling of 675A almost collapsed
675C	M.	300 ODP/3 days	2700	2	Small scrotal swelling and quilting of back. Almost mature response
677A	F.	100 ODP/day	1400	1	Large swollen pubic lobes
677C	F.	100 ODP/day	1600	1	Reddened pubic lobes and quilting of back. Almost a mature response
680A	M.	100 ODP/3 days	1800	1	Large penile and scrotal swellings. Diffuse swelling of buttocks
693A	F.	Natural pubertal swelling with roll swellings on thighs and base of tail			
698B	M.	100 EO/day	2000	2	Large scrotal and penile swellings. Thickened buttocks

Table 3. *Details of pig-tail monkeys used in experiments*

ODP, Oestradiol-3-17β dipropionate.

Specimen no.	Sex	Hormone treatment (doses in μg.) (*a*) Routine	(*b*) Total dose of hormone	No. of days since last injection	State of sexual skin
681A	M.	300 ODP/3 days	3000	2	Large circumanal and penile swellings. Scrotum flabby
682E	F.	300 ODP/3 days	1800	7	Collapsed flushed and thickened buttock skin
683B	F.	100 ODP/day	2600	1	Large circumanal and pubic swellings
683C	F.	100 ODP/day	2600	2	683B at death 24 hr. later
685A	F.	300 ODP/3 days	1800	2	Moderate circumanal and pubic swellings
686A	M.	300 ODP/3 days	3900	1	Large circumanal and penile swellings. Scrotum flabby
687A	F.	300 ODP/3 days	1800	2	Moderate circumanal and pubic-lobe swellings
688B	F.	Naturally occurring swelling in collapsing phase			
688C	F.	300 ODP/3 days	1500	2	Moderate circumanal and pubic-lobe swelling
689D	F.	300 ODP/3 days	1200	8	Moderate circumanal and base of tail swelling. Collapsing
690A	F.	300 ODP/3 days	3000	3	Large circumanal and pubic lobe
690C	F.	300 ODP/3 days	2100	3	Large circumanal and pubic-lobe swellings increasing in size
691D	F.	300 ODP/3 days	2100	9	Moderate circumanal and large pubic swellings. Collapsing

Sometimes the circumanal and pubic-lobe swellings were separated, but on other occasions the two regions were joined by swollen vulval lips. One animal (no. 682) on all occasions responded to oestrogen treatment in an atypical manner. The first response (682A) consisted of a small pubic-lobe swelling and a diffuse pink swelling of the buttocks. The last response (682E) consisted of a small pubic-lobe swelling and a hyperaemic, slightly thickened skin over the buttocks. The male pig-tail monkeys responded slowly to oestrogen by forming hard circumanal swellings completely separate from a flabby pale scrotum and a large hard swollen penis. The appearance of the naturally occurring swelling (688B) did not differ from the experimentally induced swellings.

Biopsy technique. The animals were anaesthetized with an intravenous or intraperitoneal injection of Nembutal, supplemented if necessary with ether during the operation. The majority of the specimens were obtained with scalpel and scissors, but a few specimens were taken with the cutting needle of a diathermy apparatus. Surface blood was quickly wiped from the specimens with cotton wool.

The swollen sexual skin consisted of the epidermis and a thickened dermis, which, in the deeper layers, had the appearance of jelly. Large amounts of exudate flowed freely from some specimens, e.g. those from the large pubic and scrotal swellings of the rhesus monkeys, whereas exudate could be obtained from baboon and pig-tail specimens only by cutting into small pieces and squeezing through a fabric.

Sexual-skin exudates. The exudates were obtained by cutting the tissue into small pieces and squeezing the exudate out through muslin or Terylene fabric. The exudates from baboon and most rhesus specimens were quickly obtained in this way, whereas the exudates from the pig-tail specimens were obtained only after considerable squeezing. In view of the instability of one or more of the components of the exudate (Ogston *et al.* 1939) and in order to minimize these changes, attempts were made to obtain the exudate in the cold, but the more viscous exudates gelled. All exudates were therefore obtained at room temperature as soon as possible. The less viscous exudates were freed of particulate material by centrifuging at 7000–8000*g* for 10 min. after the addition of a small amount of Hyflo Super-Cel. This procedure did not remove all particulate matter from the highly viscous exudate from pig-tail monkeys, and some error may have been introduced into some of the analyses, especially the viscosity measurements.

Preparation of mucopolysaccharide from the exudate. Several preparations of the mucopolysaccharide fraction were made, but as small amounts only of material were available the purification was not carried very far. The products are termed MP1 etc.

MP1 was prepared at room temperature by acidifying exudate from rhesus monkeys 624A with an equal volume of 2% (v/v) acetic acid. The precipitate was redissolved in $NaHCO_3$ (1%, w/v), reprecipitated with 2% (w/v) acetic acid and washed with ethanol and ether.

MP2 was prepared from 25 ml. of exudate of 609A in the same manner as MP1, followed by one treatment with Sevag's reagent, at room temperature.

MP3 was prepared from the swollen pubic lobe of 655A, by diluting the exudate with an equal volume of water and adding 4 vol. of ethanol at −20° (Ropes, Robertson, Rossmeisl, Peabody and Bauer, 1947). The voluminous precipitate was redissolved in 0·02N-NaOH. A further ethanol precipitation at −20° was carried out and the precipitate washed in ethanol and ether. The yield after drying over P_2O_5 *in vacuo* was 23 mg.

MP4 was prepared from approx. 500 g. of coarsely-minced sexual skin of baboon 615D. After removal of exudate the skin was further extracted with 200 ml. of 0·9% NaCl at 0°. The combined exudate and extract were dialysed at 0° against water and precipitation with 4 vol. of ethanol at −20° was carried out. The bulky precipitate was washed in ethanol and dried over $CaCl_2$ *in vacuo*, after which it was extracted for 60 hr. at 0° with 80 ml. of 0·1N-sodium acetate. After dialysis against water at 0°, the mucopolysaccharide fraction was precipitated from the extract with 4 vol. of ethanol at −20°. The fine precipitate was washed in ethanol and ether and dried over $CaCl_2$. Yield was approx. 10 mg.

MP5 was prepared from 150 g. of swollen circumanal skin of pig-tail specimen 683C in a similar manner to MP4, except that the precipitate that was formed by the first ethanol treatment was not dried but redissolved in water. Yield was approx. 5 mg.

MP6 was prepared by repeatedly deproteinizing (with Sevag's reagent) 15 ml. of exudate from penis and circumanal region of 681A. Further protein was removed with Lloyd's reagent at pH 2·0–3·0 (acetic acid). The very viscous supernatant was mixed with 4 vol. of ethanol at room temperature and the precipitate washed with ethanol and dried over $CaCl_2$. This material was extracted with about 20 ml. of phosphate buffer, pH 8·0 (94·5 vol. of M/15-Na_2HPO_4 and 5·5 vol. of M/15-KH_2PO_4) and 80 ml. of ethanol was added to the extract, which was not viscous. The precipitate was dissolved in water and a further ethanol precipitation carried out. After washing with ethanol and drying over P_2O_5 43 mg. of material was obtained.

Hyaluronidase preparations. Testicular hyaluronidase I was prepared from bull's testis by the method of Freeman, Anderson, Oberg & Dorfman (1949). Its potency was not determined. Testicular hyaluronidase II (1030 turbidity reducing units/mg.) was obtained from Wyeth Laboratories Inc., Philadelphia, Pa., U.S.A. It was used at concentrations of 1 mg./ml. in 0·9% NaCl.

Streptococcal hyaluronidase was a crude enzyme fraction prepared from *Streptococcus haemolyticus* by the method of Rogers (1945). It was stored in a concentrated form in glycerol at −30°.

Hyaluronic acid. The 'reference' hyaluronic acid (Allen and Hanburys Ltd.) had been prepared from acetone-dried umbilical cords by extracting with water and precipitating from the extract with ethanol saturated with potassium acetate. The water-soluble material from this precipitate was again treated with ethanol. The preparation contained 4·3% of N, 31·9% of hexosamine and 1·6% of S; it has been examined previously by filter-paper electrophoresis (Rienits, 1953*a*).

Chondroitin sulphate. This preparation was made from bovine trachea by the method of Bray, Gregory & Stacey (1944) and contained 4·72% of N and 23·2% of hexosamine.

Filter-paper chromatography. The ascending technique was used, at 18°, with Whatman filter paper no. 54. Positions of the sugars were located with aniline hydrogen

oxalate (Partridge, 1948). The most suitable solvent system was found to be pyridine–amyl alcohol–water (7:7:6, by vol.). In contrast with the reports of Werner & Odin (1949), the hexuronic acids had R_F values of about half those of the hexosamines. Individual hexosamines and hexuronic acids were not separated in this solvent. The individual hexosamines were identified by degrading with ninhydrin and identifying the resultant pentoses (Gardell, Heijkenskjold & Roch-Norland, 1950).

Determinations of viscosity. All the viscosity determinations were carried out in the same capillary microviscometer (Hardwicke & Squire, 1952) with a flow time of 22·4 sec. for the standard buffer solution. The outer tube contained 0·8 ml. of the fluid under examination and the viscometer was immersed to a standard depth in the fluid. The readings were made at 25°.

Exudates were weighed into the outer tube and the volume was brought to 0·8 ml. with standard buffer (0·2 M-NaCl and phosphate buffer, pH 7·4; as used by Ogston & Stanier, 1950). The flow time of the mixture was measured. A further 0·8 ml. of buffer was added to samples of 0·4 ml. of the mixture removed for hyaluronic acid glucosamine determination. The flow time of the fluid remaining in the viscometer was measured. Two further dilutions with buffer were carried out. A correction was made to the flow time to allow for the effect of protein in the exudate. The value of the correction was ascertained by measuring the flow times of several sera at various dilutions. The correction was calculated to be 2·5 sec. for 1 g. of protein/100 ml. of exudate or dilution.

Filter-paper electrophoresis. This was carried out as described by Rienits (1953*a*), as was the elution of material from the paper after the electrophoresis.

Chemical analyses

Hexosamine. The Elson & Morgan (1933) method, as modified by Johnston, Ogston & Stanier (1951), was used with several further modifications. Samples containing hexosamine were placed in Quickfit test tubes and evaporated to dryness with a water bath and water pump. Acidic materials (such as HCl hydrolysates) were then left *in vacuo* over NaOH overnight. If the test sample contained buffer the hexosamine standards (glucosamine hydrochloride; British Drug Houses Ltd.) were also made up in the buffer and evaporated to dryness. To this material was added 1·5 ml. of acetyl acetone reagent (0·1 ml. of acetyl acetone dissolved in 7·5 ml. of 0·5 N-Na_2CO_3). The remainder of the procedure was as described by Johnston *et al.* (1951). A set of standards was put in with each batch of unknowns.

Hexuronic acid. This was determined by the method of Ogston & Stanier (1951), a correction being used for protein (Ogston & Stanier, 1952).

Nitrogen. This was determined by the Kjeldahl digestion of Hiller, Plazin & van Slyke (1948) and distillation of the NH_3 into boric acid.

Protein. Protein in the exudates was determined by a biuret method (Wolfson, Cohn, Calvary & Ichiba, 1948).

Hyaluronic acid and chondroitin sulphate. These substances were estimated in skin specimens by a colorimetric method based on the result of Pearce & Watson (1949) that each 1 g. of human skin contained sufficient hyaluronic acid and chondroitin sulphate to yield 60 μg. of free hexosamine from each compound after acid hydrolysis. About 1 g. of skin (or rather less of swollen sexual skin) was weighed into a centrifuge tube and 2 ml. of N-NaOH and 1 ml. of water were added. The mixture was incubated at 37° for 48 hr., after which the opalescent solution was brought to pH 7·0–8·0 with 2 M-acetic acid and vigorously shaken with 5 ml. of Sevag's reagent. The denatured protein was compacted at the interface by centrifuging, and the supernatant fluid was adjusted to pH 5·0 (indicator paper) with dilute acetic acid. The denatured protein was washed with 1 ml. of acetate buffer, pH 5·0, and combined washing and supernatant shaken with 50 mg. of Lloyd's reagent. The Lloyd's reagent was washed with 1 ml. of the acetate buffer and combined washing and supernatant concentrated to 3–4 ml. over P_2O_5 *in vacuo*. Ethanol–ether (3 : 1, v/v; 30 ml.) was added and the mixture kept at −30° overnight. The precipitate was washed with 2 ml. of cold ethanol–ether and allowed to dry in warm air.

The hyaluronic acid contained in the precipitate was removed by enzymic hydrolysis. The precipitate was suspended in 1 ml. of enzyme–phosphate buffer (M/15-phosphate buffer, pH 7·0, in 0·1 M-NaCl containing in each ml. 0·02 ml. of streptococcal hyaluronidase solution), a few drops of toluene were added and the mixture was incubated at 37° for 18 hr. The small amount of precipitate remaining was centrifuged off and washed with 1 ml. of water. To the combined supernatant and washing was added 18 ml. of ethanol–ether and precipitation completed at −30° overnight. The supernatant, together with 2 ml. of ethanol–ether washing, was evaporated to dryness and heated with 2 ml. of N-HCl at 100° for 2 hr. This fraction contained the hyaluronic acid hexosamine.

The precipitate remaining after removal of the hyaluronic acid was extracted twice with 2 ml. portions of 10% (w/v) barium acetate. The chondroitin sulphate fraction was precipitated from the combined barium acetate extracts by the addition of ethanol to 20% (v/v) concentration and leaving at 0° overnight. The precipitate was washed with 1 ml. of aq. 20% ethanol, dried in air and then hydrolysed in 3 ml. of 4 N-HCl at 100° for 4 hr.

The hexosamine contents of the hydrolysates of the two fractions were determined and the hyaluronic acid and chondroitin sulphate concentrations in the skin specimens expressed as mg. of hexosamine /100 g. of skin (wet wt.). A correction was made for the contribution of hexosamine from the enzyme–buffer mixture.

The composition of the hyaluronic acid and chondroitin sulphate fractions was examined. The hyaluronic acid fraction contained no detectable acid-hydrolysable sulphate, and the hexosamine : hexuronic acid ratio varied from 0·84 to 1·39 (five determinations). The chondroitin sulphate fraction contained a molar ratio of hexosamine to uronic acid of approximately unity; the hexosamine was galactosamine, there was sulphate present after hydrolysis and paper electrophoresis showed that the hexosamine-containing material migrated at the same rate as chondroitin sulphate. Both fractions contained considerable amounts of protein. Recoveries of hyaluronic acid added at the beginning of the assay were low and variable (52, 78 and 65%). The reproducibility of the determinations on rat skin were: for hyaluronic acid 5·96 ± 0·90 mg. of hexosamine/100 g.; for chondroitin sulphate 6·90 ± 0·55 mg. of hexosamine/100 g. of skin (nine determinations). Thus the method has a low order of reproducibility but the results

obtained show that it has sufficient sensitivity to demonstrate differences in acid mucopolysaccharide content of sexual skin during its phases of turgescence and deturgescence.

Hyaluronic acid in exudates. This was estimated as mucin hexosamine. The exudates were brought to 2% with respect to acetic acid and put aside in the refrigerator overnight. The mucin was separated by centrifuging and washed with 1 ml. of 1% acetic acid. Several drops of N-NaOH were added to the mucin and the solution was transferred to a Quickfit test tube for hydrolysis in 4N-HCl for 4 hr. at 100°. Hexosamine was then determined in the hydrolysate. The hexosamine liberated in hydrolysis for 4 hr. was the same as in 6 hr. Determinations were made in duplicate and agreed to within 2%.

Sulphate. Sulphate released on hydrolysis in 2N-HCl for 2 hr. at 100° was detected turbidimetrically. The hydrolysate was centrifuged at 7000–8000*g* (for 15 min.) and to 1 ml. of the supernatant was added 1 ml. of 2% (w/v) $BaCl_2$ and 1 ml. of ethanol. After 15 min. a definite perceptible haze could be seen from material containing 50 μg. or more of SO_4^{2-} ions/ml. No attempt was made to measure the sulphate quantitatively.

RESULTS

Mucopolysaccharide of the sexual-skin exudate

Action of hyaluronidases. Exudates (0·5 ml.) from specimens 584A, 575A, 616A, 689D and 691D were diluted with 0·5 ml. of 0·9% NaCl and incubated for 18 hr. with and without streptococcal hyaluronidase (0·02 ml.) or testicular hyaluronidase (0·1 ml.). In all specimens the unstable nature of the mucin, originally observed by Ogston *et al.* (1939), was noted by the change in appearance of the mucin precipitate (on addition of acetic acid to 2%) from a clot to a turbidity after incubation without added enzyme. Incubation with either testicular or streptococcal hyaluronidase abolished the ability to form a mucin precipitate in all specimens.

Analyses of mucopolysaccharide preparations. MP1 was hydrolysed with 2N-H_2SO_4 for 4 hr. at 100°, neutralized with $Ba(OH)_2$ and brown colouring matter was largely removed with charcoal. MP2 was hydrolysed with 2N-HCl for 4 hr. at 100° and HCl removed by evaporation to dryness on the water pump. The two hydrolysates were examined for the nature of their sugar content by filter-paper chromatography. Hydrolysates from hyaluronic acid and chondroitin sulphate were also examined together with reference sugars. In both MP1 and MP2 there were only two classes of sugars present, one with an R_F that of hexosamines (0·20–0·23) and the other with an R_F of the hexuronic acids (0·08–0·10). There appeared to be much more hexosamine present on the chromatogram than hexuronic acid (owing to the destruction of the latter during acid hydrolysis). The hexosamine in MP2 was shown to be glucosamine since it formed arabinose (R_F 0·47) on oxidation with ninhydrin, in contrast with galactosamine which formed lyxose (R_F 0·67) on treatment with ninhydrin. The identity of the hexuronic acid present in MP1 and MP2 is not known since solvent systems for the separation of the individual hexuronic acids were not available.

MP6 contained less than 0·3% of total P and less than 1·5% of S (as acid-hydrolysable sulphate). Preparations MP3 to MP6 were analysed quantitatively for hexosamine, hexuronic acid and total N. The results, together with a derived value for protein, are shown in Table 4. In three of the four preparations the molar ratio of hexosamine to hexuronic acid does not differ greatly from unity. Hyaluronic acid contains equimolar quantities of hexosamine and hexuronic acid (Meyer, 1958).

Preparations MP3 and MP6 were examined by filter-paper electrophoresis. The results are shown in Fig. 1. MP6 appears to contain a single compound containing hexuronic acid but there was not sufficient material to repeat the electrophoresis with hexosamine as a basis for locating the mucopolysaccharide. MP3 appeared to consist of two compounds each containing hexosamine and hexuronic acid. Neither of these compounds migrated as far as chondroitin sulphate, which under the same conditions would have migrated approx. 32 cm. The appearance of the two peaks may have

Table 4. *Quantitative analyses of MP3–MP6*

	N (%)	Hexosamine (%)	Hexuronic acid (%)	Molar ratio Hexosamine / Hexuronic acid	Protein (%)
MP3	9·75	3·46	2·96	1·26	59·4
MP4	6·70	4·58	3·10	1·59	42·5
MP5	6·88	5·30	5·44	1·05	39·1
MP6	10·25	7·56	9·17	0·90	62·2

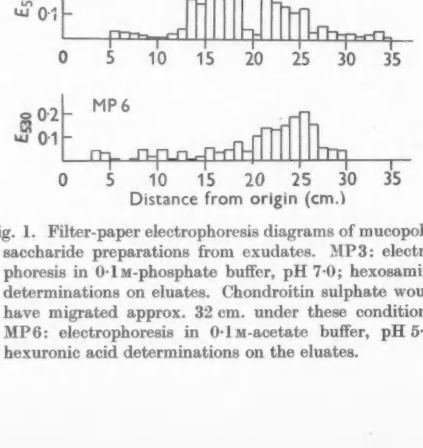

Fig. 1. Filter-paper electrophoresis diagrams of mucopolysaccharide preparations from exudates. MP3: electrophoresis in 0·1M-phosphate buffer, pH 7·0; hexosamine determinations on eluates. Chondroitin sulphate would have migrated approx. 32 cm. under these conditions. MP6: electrophoresis in 0·1M-acetate buffer, pH 5·0; hexuronic acid determinations on the eluates.

been due to faulty extraction of mucopolysaccharide from the filter-paper strips subsequent to electrophoresis or may have been due to two distinct polymer sizes of hyaluronic acid. It was not possible to decide between these possibilities owing to lack of material.

Relative viscosity measurements and hyaluronic acid of exudates

The ratio (called the 'viscosity concentration ratio' = $1/c.\log \eta/\eta_{solvent}$, where $\eta/\eta_{solvent}$ is the relative viscosity) was determined on a number of exudates and used as an indication of the degree of polymerization of the hyaluronic acid in exudates. [A similar quotient was used by Ragan & Meyer (1949) in studies on synovial fluids.] There was considerable variation observed in the value of the ratio, ranging from 0·0014 to 0·035, although about 80% of the values fell within the range 0·003–0·01. The greatest variation occurred in the material from the baboons and rhesus monkeys where the hyaluronic acid concentration is lowest. It was not possible to discern any species differences in the viscosity concentration ratio nor was any relationship detected between the ratio and the state of the sexual-skin swelling, i.e. the degree of swelling or the phase of the cycle. There did not seem to be any relationship between the concentration ratio and the concentration of the hyaluronic acid in the exudate.

Concentrations of acid mucopolysaccharides in sexual skin

In Tables 5–7 are set forth the results of analyses for water, chondroitin sulphate and hyaluronic acid together with the exudate hyaluronic acid levels from the sexual skins of the three species. The water content of the sexual skin increases enormously when the tissue swells and reaches well over 90% in some instances. In all the species the hyaluronic acid concentration of the swollen skin is greater than that of unswollen sexual skin or ordinary skin. The reverse is seen with the chondroitin sulphate concentrations. When the concentrations of the acid mucopolysaccharides are expressed in terms of dry weight of tissue it can be seen that the chondroitin sulphate concentration is, with few exceptions (e.g. 616F, 688C and 690A), slightly greater in swollen sexual skin than in unswollen skin. This increase in concentration occurs despite the increase during swelling in solid matter of the sexual skin due to an influx of electrolyte and serum protein; thus the total amount of chondroitin sulphate present in a sexual skin increases when it swells.

There are marked species differences in the concentrations of hyaluronic acid in the swollen sexual skins, and also there are marked differences in the exudate hyaluronic acid concentrations from the three species. The concentrations of hyaluronic acid in swollen sexual skin and exudate may determine to some extent the turgidity of the skin. Thus in general the swollen sexual skins of the pig-tail monkeys were very hard and these had high concentrations of hyaluronic acid in both whole sexual skin and exudate. The specimens 682E and the scrotal swelling of 681A (Table 7) were not hard (see Materials and Methods section), and the hyaluronic acid concentrations in exudates from these two tissues were lower than exudates from other pig-tail monkey sexual skins. In addition, the total tissue hyaluronic acid values were lower than most other pig-tail specimens of sexual skin. In the rhesus monkeys (Table 6) the one hard swelling (the roll swelling from the thigh of 693A) gave much higher values for exudate hyaluronic acid and total tissue hyaluronic acid than the other specimens which were of the softer, more flabby variety. Within the rhesus group (Table 6) specimens 675A, B and C illustrate the manner in which the sexual-skin response of rhesus monkeys alters with successive courses of oestrogen, and 677A and C illustrate the same point in a less complete fashion. The oedema is less, and less hyaluronic acid is produced. The results from 673B and perhaps 677C may be taken as indicating that the acid mucopolysaccharide content of unswollen sexual skin is not different from that of ordinary skin.

In all species there was a relationship between the water content of the tissues and their hyaluronic acid contents. With the baboons there is a suggestive, but insignificant, correlation between the water and the hyaluronic acid concentrations, but there is a significant relationship between the water concentration and the logarithm of the hyaluronic acid concentration (r 0·645; P lies between 0·05 and 0·02). The analyses from the rhesus monkeys show similar relationships, although in this series there was a highly significant correlation between the water content and the logarithm of the hyaluronic acid concentration (r 0·5975; P lies between 0·01 and 0·001). The analyses from the sexual skins of the pig-tail monkeys reveal that there is a highly significant correlation between the water content and the hyaluronic acid concentration or its logarithm (r 0·786 and r 0·923 respectively with P less than 0·001 in both cases).

The data of Table 5 show that in the baboon the level of hyaluronic acid in the exudate is much lower in the turgescent phase than during the deturgescent phase. Thus values for the deturgescent phase are 5·1, 7·9 and 11 and for the turgescent phase are 0·9, 2·3, 2·6, 1·8, 3·0, 2·4 and 3·5. The lowest value for exudate hyaluronic acid is from a very early swelling (616C) and the highest from an

almost collapsed swelling (616D). Similar differences between the turgescent and deturgescent phases were not seen in the exudates of the other species studied.

From the data in Table 5 it is obvious that in the sexual skin of the baboon with the exception of 616D only a proportion of the tissue hyaluronic acid could be in the exudate. To illustrate this a hypothetical 'hyaluronic acid space' was calculated to represent the volume of exudate required by each 100 g. of tissue if the hyaluronic acid of the tissue was solely present in the exudate. Values of

Table 5. *Hyaluronic acid and chondroitin sulphate concentrations in circumanal sexual skins from baboons*

Specimen no.	Condition of sexual skin	Water content (%, w/w)	Concn. in skin: Hyaluronic acid (mg. of hexosamine/100 g. of tissue) Wet	Hyaluronic acid Dry	Chondroitin sulphate Wet	Chondroitin sulphate Dry	Concn. of protein in exudate (g./100 ml.)	Concn. of hyaluronic acid in exudate (mg. of glucosamine 100 ml.)	Hyaluronic acid space* (ml./100 g.) of tissue)
615B	Slightly swollen	90·7	6·0	64	1·6	17	—	—	—
616C	Very early swelling	90·8	5·1	56	2·1	23	3·3	0·9	590
616G	Rapidly increasing in size	88·3	4·6	39	2·5	21	3·5	2·3	210
616L	Late turgescent phase	—	—	—	—	—	3·3	—	—
616K	Almost fully swollen	94·0	18	300	—	—	2·0	2·6	690
616F	Fully swollen	93·3	13	200	0·8	12	3·8	1·8	740
616H	Fully swollen	93·8	14	230	2·0	32	5·4	3·0	480
616E	Fully swollen	92·9	11	150	2·4	34	4·2	2·4	450
621A	Fully swollen	—	—	—	—	—	—	3·5	—
615D	Commencing to collapse	95·7	8·7	200	2·1	49	2·8	5·1	170
616J	Collapsing	92·5	12	160	2·9	39	1·8	7·9	160
616D	Almost collapsed	83·4	6·2	37	3·5	21	2·6	11	60

* Hyaluronic acid space = $\frac{\text{Concn. of hyaluronic acid in skin}}{\text{Concn. of hyaluronic acid in exudate}}$.

Table 6. *Hyaluronic acid and chondroitin sulphate relationships in skin from rhesus monkeys*

Specimen no.	Condition of sexual skin	Water content (%, w/w)	Concn. in skin: Hyaluronic acid (mg. of hexosamine/100 g. of tissue) Wet	Hyaluronic acid Dry	Chondroitin sulphate Wet	Chondroitin sulphate Dry	Concn. of hyaluronic acid in exudate (mg. of glucosamine/100 ml.)	Hyaluronic acid space* (ml./100 g. of tissue)
673A	Swollen pubic lobe	82·4	14	81	0·8	4·5	—	—
673B	Collapsed pubic lobe	51·0	5·8	10	4·2	8·6	—	—
675A	Large swollen scrotum	—	15	—	1·2	—	—	—
675B	Swollen scrotum: collapsing	—	7·1	—	0·8	—	—	—
675C	Skin of chest	69·1	4·4	14	—	—	—	—
675C	Scrotum: almost collapsed	71·7	16	—	—	—	—	—
675C	Base of tail swelling	86·5	8·5	63	—	—	5·5	160
675C	Quilted skin of thighs	77·1	10	44	—	—	no exudate	—
677A	Large swollen pubic lobe	90·4	12	128	1·4	15	—	—
677C	Skin of chest	50·9	6·0	12	—	—	—	—
677C	Flushed pubic lobe	68·5	5·4	17	—	—	—	—
677C	Slightly quilted thigh skin	71·4	4·6	16	—	—	—	—
677C	Small swelling at tail base	70·2	9·4	32	—	—	—	—
680A	Skin of chest	64·0	3·8	11	7·6	21	—	—
680A	Thickened skin of buttocks	92·6	8·1	110	2·2	30	1·5	530
680A	Grossly swollen scrotum	93·2	9·5	139	2·5	37	1·4	680
693A	Hard roll swelling	84·3	33	210	2·7	17	99	33
698B	Skin of chest	63·0	5·2	14	6·2	17	—	—
698B	Swollen penile sheath	—	11	—	1·0	—	5·6	200
698B	Thickened skin of flanks	80·0	14	72	3·2	16	10	140
698B	Flabby scrotal swelling	82·0	11	63	6·1	34	2·0	560

* See footnote to Table 5.

more than 100 ml./100 g. of tissue show that all the hyaluronic acid could not possibly be present in the exudate. In all species this calculation demonstrates that part of the tissue hyaluronic acid is not contained in the exudate. Attempts were made to fractionate the tissue hyaluronic acid into that which was easily extractable and that which resisted extraction. Several methods of extraction were used and these together with the results obtained are presented in Table 8. In all the specimens a proportion of the hyaluronic acid was not extracted. This may have been due partly to incomplete extraction by the methods used but is probably an indication of the hyaluronic acid which

Table 7. *Hyaluronic acid and chondroitin sulphate relationships in skin from pig-tail monkeys*

Specimen no.	Condition of sexual skin	Water content (%, w/w)	Concn. in skin: Hyaluronic acid (mg. of hexosamine/100 g. of tissue) Wet	Hyaluronic acid Dry	Chondroitin sulphate Wet	Chondroitin sulphate Dry	Concn. of hyaluronic acid in exudate (mg. of glucosamine/100 ml.)	Hyaluronic acid space* (ml./100 g. of tissue)
681A	Skin of chest	57·4	4·4	10	8·5	20	—	—
681A	Large circumanal swelling	94·9	60	1200	2·8	55	69	87
681A	Swollen penis	96·2	62	1600	1·2	32	63	98
681A	Large flabby scrotum	96·6	21	620	1·8	53	12	170
682E	Skin of chest	60·0	4·3	11	7·9	20	—	—
682E	Thickened circumanal region	77·8	14	63	5·2	23	1·8	770
682E	Swollen base of tail	88·8	16	140	5·7	51	11	150
683B	Large circumanal swelling	93·9	48	790	2·1	34	93	52
683B	Large pubic swelling	91·5	38	450	2·1	25	55	69
685A	Large circumanal swelling	91·5	60	710	1·5	18	50	120
685A	Large pubic swelling	93·6	50	800	1·2	19	29	170
687A	Large circumanal swelling	92·8	47	640	2·4	33	23	210
687A	Large pubic swelling	91·9	42	510	1·6	20	23	180
688B	Moderate circumanal swelling	—	59	—	5·5	—	—	—
688C	Skin of chest	50·0	3·9	78	6·1	12	—	—
688C	Large circumanal swelling	92·9	72	1000	0·9	13	—	—
688C	Large pubic swelling	92·0	51	630	1·3	16	—	—
689D	Skin of chest	60·0	7·9	20	—	—	—	—
689D	Moderate circumanal swelling	93·2	41	600	—	—	85	49
689D	Small pubic lobe	82·5	27	150	—	—	—	—
690A	Large circumanal swelling	95·1	47	960	1·6	33	130	36
690A	Large pubic swelling	94·6	60	1100	2·0	37	130	46
690C	Skin of chest	67·0	5·2	16	5·5	17	—	—
690C	Large circumanal swelling	93·8	41	660	3·2	52	59	69
690C	Large pubic swelling	91·3	47	540	3·0	35	62	76
691D	Skin of chest	62·1	6·1	16	—	—	—	—
691D	Moderate circumanal swelling	91·1	22	250	—	—	18	120
691D	Fair pubic swelling	92·3	18	240	—	—	33	55

* See footnote to Table 5.

Table 8. *Effect of various extraction procedures on acid mucopolysaccharide levels in sexual skin*

Specimen no.	Concn. of acid mucopolysaccharide: Before extraction: Hyaluronic acid hexosamine	Before extraction: Chondroitin sulphate hexosamine	After extraction: Hyaluronic acid hexosamine	After extraction: Chondroitin sulphate hexosamine
	(mg./100 g. original wet wt. of tissue)			
615D*	8·7	2·1	5·5	1·7
616H*	14·3	2·0	13·2	1·3
677A†	12·3	1·4	9·3	1·2
676A†	9·3	1·2	4·2	1·0
686A‡	40·2	2·3	6·2	0·9

* Chopped into small pieces and extracted with 0·9% NaCl until no more acid-precipitable material was obtained.
† Dried in acetone, ground with sand and extracted with 10% (w/v) sodium acetate.
‡ Homogenized in a stainless-steel Potter–Elvehjem homogenizer in 0·9% NaCl. The supernatant, on centrifuging at 7000*g* for 15 min., was still turbid and may have contained finely divided particulate matter.

was bound to insoluble structures in skin. In all cases the level of chondroitin sulphate fell upon extraction and this was probably due to the loss of particulate matter upon extraction.

DISCUSSION

The properties of the mucopolysaccharide in the sexual-skin exudate are consistent with the material being hyaluronic acid. This confirms the expectations based on the observations of Chain & Duthie (1940) and Duran-Reynals *et al.* (1950), who showed that a hyaluronidase-sensitive material was present in exudate and extracellular spaces of swollen sexual skin. In addition hyaluronic acid is a component of rat and human skin (Meyer & Chaffee, 1941; Pearce & Watson, 1949). Swollen sexual skin, however, differs from ordinary skin in that part of the hyaluronic acid appears in a soluble form and is not all bound in a form which resists extraction except with drastic procedures such as dilute alkali. Results in Tables 5–7 show that this exudate hyaluronic acid represents only a part of the general increase in this substance which occurs in sexual skin when it swells. The same tables also permit of the conclusion that during turgescence the total quantity of chondroitin sulphate also increases, although only slightly, in sexual skin when it swells. None of this chondroitin sulphate was found in the exudate and the role of chondroitin sulphate in the sexual-skin swelling was not further investigated.

Results in Tables 5–7 demonstrate a relationship between the hyaluronic acid content of sexual skin and its water content. This appears to be the first time that such a relationship has been obtained for any tissue with a semi-quantitative analytical method. As noted in the section on Materials and Methods the analyses for the mucopolysaccharides were not very satisfactory but were, however, sufficient to demonstrate gradations of hyaluronic acid content existing between unswollen and swollen sexual skin. The reason for this association between total water content of the tissue and its hyaluronic acid content is not understood and is made difficult by the fact that the sexual-skin oedema is both intra- and extra-cellular in composition (Aykroyd & Zuckerman, 1938), and hyaluronic acid is found extracellularly by histochemical techniques (Duran-Reynals *et al.* 1950). It would seem that hyaluronic acid production is but one factor in bringing about oedema in the sexual skin. The following considerations refer only to a possible mechanism whereby hyaluronic acid could play a part in regulating extracellular-fluid volume.

Among the animals examined in this study only the baboon exhibited regular and consistent sexual-skin cycles. In the baboon the cycle was consistently characterized by a moderately slow turgescence followed by a sudden deturgescence. The cycle in the other species, which had to be experimentally induced, was drawn out and irregular. It is only in material from the baboons that analyses show any regular pattern (Table 7). During the turgescent phase the protein content of the exudate increased and the level of hyaluronic acid in the exudate remained low. Immediately after the onset of deturgescence and subsequently, the exudate protein concentration fell and the hyaluronic acid content of the exudate rose to much higher levels than during the turgescent phase. The concentration of protein found in the exudate of the turgescent phase suggests that the permeability to serum protein of the blood capillaries in the sexual skin is increased during the turgescent phase. However, preliminary experiments (Rienits, 1953*b*) did not show any significant variation during the various phases of the sexual-skin cycle of the rate of transfer of ^{131}I-labelled serum protein from blood to exudate.

These observations are consistent with a hypothesis which assumes that the hyaluronic acid which is not present in the exudate, and which gives evidence of being 'bound' or in an insoluble form, exerts a control on the passage of plasma proteins from the vascular to the lymph capillaries. During the turgescent phase the increasing quantities of this 'bound' hyaluronic acid would cause an accumulation of plasma proteins in the extracellular phase by hindering their access to the lymph capillaries. These proteins would attract fluid to satisfy the new osmotic relationship with the blood (in accordance with the Starling concept) and exudate would appear. The removal of hyaluronic acid from its non-exudate location would allow the removal of the plasma proteins in the exudate to the lymph and deturgescence would follow. Deturgescence in the baboon is a rapid process and the high exudate hyaluronic acid concentration during this phase is suggested as being derived from the bound or insoluble hyaluronic acid which has been liberated rapidly into the exudate but not removed from the exudate at such a rapid rate.

The hypothesis does not require any changes in capillary 'permeability' to occur during the sexual-skin cycle. In addition to the observations of Rienits (1953*b*) mentioned above, the experiments of Duran-Reynals *et al.* (1950) suggest that there is no altered permeability of sexual skin during turgescence. These authors caused the collapse of certain regions of swollen sexual skin by slight pressure after infiltration with hyaluronidase. The swellings did not swell again until the next sexual-skin cycle. Hyaluronidase is not known to affect

capillaries more than transiently (Zweifach & Chambers, 1950) and the effect is to increase permeability.

Day (1952) has produced evidence that hyaluronic acid is a prime factor in the regulation of the passage of fluid through membranes made of sheets of subcutaneous fascia. Hyaluronic acid was regarded as the natural material which reduced the pore size in the meshwork of fibres embedded in a ground substance of protein and mucopolysaccharides. Admittedly the results of the experiments of Day (1952) performed *in vitro* are difficult to transpose to conditions *in vivo*, but it seems possible that hyaluronic acid could also regulate the flow of proteins as well as fluid through extracellular regions.

The failure to find any consistent patterns of changes in the 'viscosity concentration ratio' during the sexual-skin cycle may indicate the lack of importance of exudate hyaluronic acid in maintaining extracellular-fluid volume. On the other hand, the work of Johnston (1955) suggests that intrinsic viscosity measurements, which are probably better measurements than those employed here, are not sensitive indicators of changes in molecular size, etc. of hyaluronic acid.

The analyses of the material from the pig-tail and rhesus monkeys revealed no pattern of events such as occurred in the baboons. It is felt that this is due to the irregular responses obtained with these species and also to the slowness with which deturgescence took place on ceasing oestrogen administration, allowing a state approaching equilibrium to be obtained in the exudate. The rapidity of deturgescence in the baboon probably resulted in the constituents of the tissue being far from an equilibrium and hence the differences observed between the concentration of the various constituents during turgescence and deturgescence. An additional factor is that deturgescence of a naturally occurring swelling is not simply due to a lack of oestrogen but that there is an effect of progesterone produced after ovulation, and this substance promotes deturgescence (Gillman, 1940; Gillman & Stein, 1941).

SUMMARY

1. An examination was made of the 'mucoprotein' present in the exudates of oedematous sexual skins of rhesus monkeys (*Macaca mulatta*), pig-tail monkeys (*Macaca nemestrina*) and baboons (*Papio papio*). The mucoprotein was susceptible to the action of both testicular and streptococcal hyaluronidase and, when partially purified, was found to contain considerable amounts of protein together with glucosamine and a hexuronic acid (presumed to be glucuronic acid) in approximately equimolar amounts. The partially purified material contained no detectable sulphate. From these observations and behaviour on paper electrophoresis it was concluded that the mucopolysaccharide in sexual-skin exudates was hyaluronic acid and that chondroitin sulphate was not present.

2. Determinations were made of the hyaluronic acid and chondroitin sulphate concentrations in sexual skin during the swelling and collapsing stages. There were considerable species differences in amounts, but in all three species swelling was accompanied by considerably increased hyaluronic acid concentration and decreased chondroitin sulphate concentration (expressed on a wet-weight basis). It was apparent that the swelling of sexual skin was accompanied by a vast increase in the absolute amount of hyaluronic acid in the tissue and calculations on a dry-weight basis revealed that the total amount of chondroitin sulphate increased slightly during the sexual-skin swelling.

3. Calculation, and extraction experiments, revealed that a considerable proportion of the hyaluronic acid of swollen sexual skin is not contained in the exudate.

4. Significant relationships were found to exist in all species between the total hyaluronic acid content of sexual skins and their water content.

5. Viscosity measurements did not reveal any evidence of any consistent changes in the degree of polymerization of the exudate hyaluronic acid during the sexual-skin cycle or reveal any relationship of degree of polymerization to hyaluronate concentration in the exudate.

6. The relationship of the changes in hyaluronic acid concentration to the oedema formation is discussed.

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The Mechanism of Action of Hydrocortisone on Mitochondrial Metabolism

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Many studies on the metabolic effects of steroids have been reported. The literature has been adequately reviewed by Gordan, Bentinck & Eisenberg (1951), Hayano & Dorfman (1951) and Lieberman & Teich (1953). Steroids have been shown to inhibit the metabolism of hexoses, phosphorylated glycolytic intermediates, tricarboxylic acid substrates and amino acids and the activity of hexokinase, tyrosinase, urease, ascorbic acid oxidase, D-amino acid oxidase, hyaluronidase, lipase and transaminase.

This study was concerned particularly with the influence of steroids on oxidative metabolism. In general, steroids inhibit the activity of enzymes requiring pyridine nucleotides for electron transfer (Guidry, Segaloff & Altschul, 1952; Sourkes & Heneage, 1952; Cochran & Du Bois, 1954). The succinoxidase system, which does not include a pyridine nucleotide, is unaffected by most steroids.

The aims of this study were to investigate the effect of 17-hydroxycorticosterone (hydrocortisone) on mitochondrial oxidation systems and to determine the mechanism of inhibitions observed. A preliminary account has been published (Gallagher, 1958).

METHODS

Enzyme preparations. Rats were killed by a blow on the neck and sheep by severing the cervical vessels. Tissues were removed rapidly and immersed in ice-cold 0·25 M-sucrose. Homogenates were prepared in 0·25 M-sucrose with glass homogenizers and fractionated by differential centrifuging at 0° (Schneider, 1948). Mitochondria were resuspended in 0·25 M-sucrose unless otherwise indicated.

Hexokinase was prepared from yeast by the Cross, Taggart, Covo & Green (1949) modification of the method of Berger, Slein, Colowick & Cori (1946). The fraction precipitated between 25 and 40% (by vol.) of ethanol was dissolved in aq. 1% glucose and stored in a frozen condition. The optimum addition of this solution for oxidative phosphorylation as determined by assay was 0·01 ml./1·5 ml. final volume.

Reagents. Adenosine triphosphate, adenosine 5-monophosphate, cytochrome *c*, diphosphopyridine nucleotide (DPN), nicotinamide, reduced glutathione (GSH), citrate, L-glutamate, succinate, L-malate, octanoate, ethylenediaminetetra-acetic acid, semicarbazide hydrochloride and inorganic reagents were obtained commercially. Succinic acid was recrystallized three times from hot water, then dissolved, neutralized and precipitated as the sodium salt with ethanol. The sodium succinate was then redissolved and crystallized from 80% ethanol. Octanoic acid was purified by distillation *in vacuo*. Cytochrome *c* was

Table 1. *Effect of hydrocortisone on oxidative metabolism by rat-liver mitochondria*

The following system was used: Adenosine monophosphate (mM); $MgSO_4$ (6·7 mM); KCl (25 mM); sodium potassium phosphate buffer, pH 7·4 (13·3 mM); cytochrome *c* (10 μM), ethylenediaminetetra-acetic acid (mM), mitochondria equivalent to 50 mg. of fresh liver, except for succinate oxidation (25 mg.) added in 0·25 ml. of 0·25M-sucrose; water to 1·5 ml.; 0·1 ml. of 20% KOH in the centre well to absorb CO_2; gas phase, air; temperature, 38°; equilibration period, 10 min. The results given are the means of all experiments.

Additions	No. of experiments	Oxygen uptake (μl./20 min.) Control	Ethanol	Hydrocortisone 1·0 mM	0·5 mM	0·1 mM
Citrate (10 mM)	6	37	39	19	21	32
Succinate (33 mM)	5	51	48	53	—	—
L-Malate (10 mM)	5	29	28	12	13	19
L-Glutamate (10 mM)	5	40	40	24	25	34
Octanoate (1·67 mM) + L-malate (0·67 mM)	8	49	49	28	32	36

standardized by the method of Potter (1957). Substrates were used as sodium salts. Sodium potassium phosphate buffer, pH 7·4, was prepared by the method of Umbreit (1957). Hydrocortisone, a commercial product free from contamination with heavy metals or calcium, was dissolved freshly before use in a minimal volume of re-distilled ethanol. The ethanolic solution could then be diluted with water to the required concentration. Control reactions with the same addition of ethanol only were carried out with each experiment.

Estimations. Oxygen uptake was measured with Warburg manometers. Inorganic phosphorus was determined by the method of Fiske & Subbarow (1925).

Spectrophotometric measurements. These were made with a Beckman model DU instrument.

RESULTS

Mitochondrial oxidations. Hydrocortisone selectively inhibits oxidative metabolism *in vitro* by liver mitochondria from normal rats (Table 1). Concentrations of the steroid above 0·1 mM inhibit the oxidation of citrate, L-malate, L-glutamate and octanoate. However, the succinoxidase system is not inhibited at mM-hydrocortisone. Ethanol in the concentration used to dissolve the steroid is not inhibitory.

Oxidative phosphorylation. Oxidative phosphorylation by liver mitochondria with L-glutamate as substrate is not affected significantly by 1·0 mM-hydrocortisone, although the rate of oxidation is reduced to nearly one-half the control rate (Table 2).

Effect of respiratory cofactors. Selective inhibition of the pyridine nucleotide-dependent enzymes, as opposed to succinoxidase, which does not require a pyridine nucleotide, suggests that the inhibitory mechanism might involve inactivation or loss of coenzymes. Alternatively, physical disruption of the mitochondria would give the same pattern of inhibition with loss of activity of enzymes other than succinoxidase and cytochrome oxidase. The two latter enzyme systems are associated with

Table 2. *Effect of hydrocortisone on oxidative phosphorylation*

The system was as in Table 1 + L-glutamate (10 mM); glucose (33 mM); hexokinase in 1% glucose (0·01 ml.); NaF (10 mM); mitochondria equivalent to 100 mg. of fresh rat liver in 0·25 ml. of 0·25M-sucrose; equilibration period, 10 min.; duration of experiment, 30 min. The results given are the means of two experiments.

Additions	O_2 used (μg.atoms) *A*	P esterified (μg.atoms) *B*	*B*/*A*
None	12·2	22·5	1·8
Hydrocortisone (mM)	6·4	9·5	1·5

mitochondrial membranes, the insoluble portion of mitochondria, and are resistant to physical treatments which inactivate other enzymes (Cleland & Slater, 1953; Watson & Siekevitz, 1956).

If the inhibition were secondary to removal of pyridine nucleotides it should be possible to prevent or reverse the effect by the addition of the appropriate nucleotide in excess. Partial reversal of hydrocortisone inhibition of L-malate oxidation was achieved by adding DPN to a concentration of mM in 0·04M-nicotinamide.

However, satisfactory prevention of hydrocortisone inhibition could not be attained unless GSH was added with the required coenzyme. Significant reversal of L-malate inhibition was provided by the addition of 0·5 mM-DPN, 0·04M-nicotinamide and 0·67 mM-GSH, but the degree of protection varied greatly from experiment to experiment. Better and less variable reversal at this level of cofactor supplementation was obtained with sheep- than with rat-liver enzymes. More reliable and complete prevention of inhibition of rat-liver enzymes was obtained by supplementing the reaction mixture with mM-DPN (in 0·04M-nicotinamide) and 1·3 mM-GSH (Table 3). Inhibition of octanoate oxidation was reversed more completely when 0·22 mM-coenzyme A was also added.

Pre-incubation of enzyme and substrate for 30 min. before the addition of hydrocortisone did not prevent the inhibition, which developed very rapidly and was almost completely established in the first 10 min. period (Table 4). A period of 5 min. equilibration was allowed after adding the steroid. Pre-incubation of substrate, enzyme and a supplement of the appropriate respiratory cofactors almost completely prevented the inhibitory effect of hydrocortisone (Table 4). The protection afforded by cofactors in this system was less variable and more complete from experiment to experiment than when the steroid was added to the reaction mixture without pre-incubation of the enzyme and substrate.

Mechanism of inhibition. It was thus established that hydrocortisone inhibits oxidations by dehydrogenases dependent on pyridine nucleotides for electron transfer and that such inhibition can be prevented by the addition of an appropriate supplement of respiratory cofactors. The problem now resolved itself to determining whether (*a*) the steroid acted directly on pyridine nucleotides and inactivated them by destruction or by the formation of complexes, or (*b*) the soluble cofactors were lost from the enzyme systems secondary to alteration of mitochondrial structure.

Loss of respiratory cofactors from the mitochondrial enzyme systems would occur readily if hydrocortisone increased the permeability of

Table 3. *Effect of respiratory cofactors on hydrocortisone inhibition*

The system was as in Table 1; mitochondria equivalent to 50 mg. of fresh rat liver were added in 0·25 ml. of 0·25 M-sucrose. The cofactor supplement for citrate and L-malate oxidations was: DPN (mM); nicotinamide (0·04 M); GSH (1·3 mM). The cofactor supplement for octanoate oxidation was: DPN (mM); nicotinamide (0·04 M); CoA (0·22 mM); GSH (1·3 mM). The results given are the means of all experiments.

Additions	No. of experiments	Oxygen uptake (μl./20 min.) Control	Hydrocortisone 1·0 mM	Hydrocortisone 0·5 mM
Citrate (10 mM)	5	29	13	18
Citrate (10 mM) + cofactors	5	32	25	30
L-Malate (10 mM)	3	22	—	12
L-Malate (10 mM) + cofactors	3	27	—	21
Octanoate (1·67 mM) + L-malate (0·67 mM)	5	49	—	28
Octanoate (1·67 mM) + L-malate (0·67 mM) + cofactors	5	54	—	42

Table 4. *Effect of pre-incubation of substrate, enzyme and cofactors on hydrocortisone inhibition of oxidations by rat-liver mitochondria*

The system was as in Table 3. Hydrocortisone was added from the side-arm compartments after 30 min. incubation: equilibration for 5 min. was allowed after mixing. The results given are the means of all experiments. — indicates 'not measured'.

Additions	No. of experiments	Time (min. after mixing–equilibration)	Oxygen uptake (μl./10 min. interval) Control	Hydrocortisone 1·0 mM	Hydrocortisone 0·5 mM
Citrate (10 mM)	4	10	18	5	10
		20	17	3	9
		30	17	2	7
Citrate (10 mM) + cofactors	4	10	23	16	21
		20	22	15	21
		30	23	16	21
L-Malate (10 mM)	2	10	12	—	8
		20	10	—	6
		30	11	—	6
L-Malate (10 mM) + cofactors	2	10	17	—	14
		20	16	—	14
		30	16	—	15
Octanoate (1·67 mM) + L-malate (0·67 mM)	4	10	18	—	9
		20	20	—	11
		30	18	—	11
Octanoate (1·67 mM) + L-malate (0·67 mM)	4	10	20	—	18
		20	21	—	17
+ cofactors		30	19	—	16

mitochondrial membranes. An active measure of mitochondrial-membrane permeability may be obtained by following spectrophotometrically the rate of reduction of DPN added externally to a system in which intact mitochondria are oxidizing an exogenous substrate. The rate of reduction of DPN may be measured readily at 340 mμ, the absorption peak of reduced DPN, and is directly proportional in intact mitochondria to the rate of entry of DPN and substrate into the particles. The passage of DPN and substrate through the mitochondrial membranes is in turn proportional to the permeability of the membranes. Thus the rate of reduction of DPN gives a direct measure of membrane permeability (cf. Christie & Judah, 1954; Gallagher, Judah & Rees, 1956).

The permeability of mitochondria in the presence of hydrocortisone was assayed by the spectrophotometric method of Christie & Judah (1953), with L-malate as substrate and mitochondria in 0·25 M-sucrose as enzyme. Fig. 1 shows clearly that hydrocortisone greatly accelerates the rate of increase of extinction at 340 mμ ($E_{340\ m\mu}$). The effect is immediate with 2 mM-hydrocortisone and becomes apparent after a short time lag with mM- and 0·5 mM-hydrocortisone. Ethanol in the concentration added with hydrocortisone does not influence the rate. The $E_{340\ m\mu}$ due to hydrocortisone in the complete system (except for L-malate) remains constant and is equal to the initial differences in $E_{340\ m\mu}$.

Suspension of mitochondria in water eliminates the barriers to the passage of DPN and L-malate through mitochondrial membranes and allows full expression of dehydrogenase activity in the presence of excess of substrate and electron acceptor (Christie & Judah, 1953). This provided a simple method with which to assess the importance of the various mechanisms that might accelerate the increase in $E_{340\ m\mu}$. Fig. 2 shows that, when the semi-permeability barriers of mitochondrial membranes have been abolished by

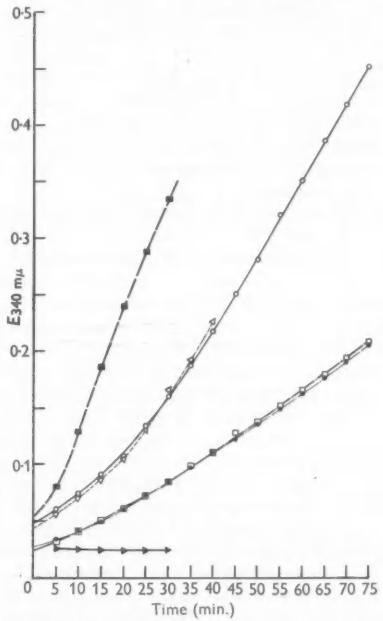

Fig. 1. Reduction of DPN by rat-liver mitochondria in 0·25 M-sucrose. The system was: sodium potassium phosphate buffer, pH 7·8 (33 mM); KCl (25 mM); $MgSO_4$ (6·7 mM); semicarbazide hydrochloride (neutralized) (0·17 M); DPN (1·5 mM); KCN (0·2 mM); 50 μl. of 1% mitochondria in 0·25 M-sucrose; final volume, 3 ml.; gas phase, air; temperature, 22°. ●, 10 mM-L-Malate; □, 10 mM-L-malate + ethanol; ■, 10 mM-L-malate + 2 mM-hydrocortisone; ○, 10 mM-L-malate + mM-hydrocortisone; △, 10 mM-L-malate + 0·5 mM-hydrocortisone; ▲, mM-hydrocortisone.

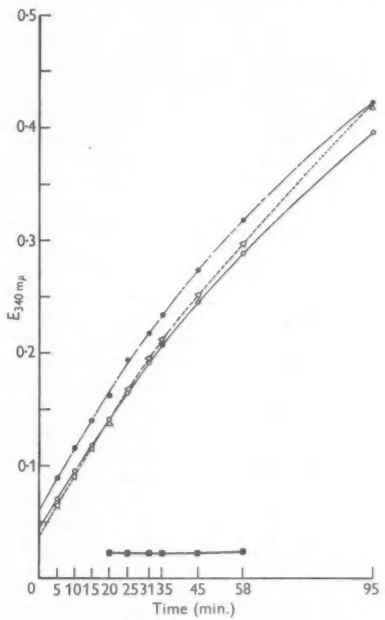

Fig. 2. Reduction of diphosphopyridine nucleotide by rat-liver mitochondria in water. The system was as in Fig. 1 except that 50 μl. of 0·5% mitochondria in water was used. ○, 10 mM-L-Malate; △, 10 mM-L-malate + ethanol; ●, 10 mM-L-malate + mM-hydrocortisone; ■, mM-hydrocortisone.

suspension of the particles in water, the addition of hydrocortisone has no effect on the rate of increase of $E_{340\,m\mu}$. Thus the rapid increase of $E_{340\,m\mu}$ exhibited by mitochondria suspended in 0·25 M-sucrose in the presence of hydrocortisone is due to an increased rate of reduction of DPN secondary to greater ease of access of either or both the nucleotide and substrate across mitochondrial membranes. The permeability of mitochondrial membranes is increased greatly by hydrocortisone. A comparison of the two figures will show that, at the highest concentration (2 mM) of hydrocortisone used, membrane permeability is increased as much as by disrupting the particles with water.

Hydrocortisone, therefore, inhibits oxidative metabolism by mitochondria *in vitro* by destroying the selective semi-permeability of mitochondrial membranes. Soluble cofactors of respiration, especially pyridine nucleotides and, to a less extent, glutathione, are leached rapidly from the particles and proportionally inactivate the nucleotide-dependent dehydrogenases. The steroid has no direct effect on dehydrogenases.

DISCUSSION

In common with steroids generally, hydrocortisone has been shown to inhibit oxidative metabolism *in vitro*. The inhibitory effect is specific to those dehydrogenases which require pyridine nucleotides for electron transfer. Similar observations for other steroids were made by Guidry *et al.* (1952), Sourkes & Heneage (1952) and Cochran & Du Bois (1954).

The efficiency of oxidative phosphorylation is not lowered, even though the rate of oxidation of the substrate used is greatly inhibited. This agrees with the finding of Clark & Pesch (1956) that, although cortisone inhibited the oxidation of α-oxoglutarate, P/O ratios were normal. Similarly, Wade & Jones (1956) found that of five steroids tested, only progesterone uncoupled oxidative phosphorylation and activated adenosine triphosphatase.

The studies of Eik-Nes, Schellman, Lumry & Samuels (1954), Westphal (1955, 1957) and others have shown that steroids can combine with protein; this gives rise to the possibility that adsorption on enzyme protein *in vitro* might lead to inhibition. However, the present study suggests that hydrocortisone does not affect the dehydrogenases directly but inactivates the enzyme systems by depriving them of the soluble, respiratory cofactors, in particular the pyridine nucleotides.

Evidence that steroids can form complexes with nucleotides has been presented by Munck, Scott & Engel (1957). Complex formation seemed possibly the mechanism of inhibition. On the other hand, better protection against hydrocortisone inhibition was achieved when GSH as well as DPN was added to the oxidizing system, and this favoured the loss of soluble cofactors from mitochondria as a likely inhibitory mechanism. It was of course possible that hydrocortisone also formed complexes with or inactivated SH groups. Anderson, Wiesel, Hillman & Stumpe (1951) suggested that deprivation of SH is the process by which cortisone inhibits the hyaluronidase system. Similarly Bacila & Guzman-Barron (1954) suggested that steroids inhibit hexokinase by combining with the SH groups of the enzyme.

The inhibitory effect of hydrocortisone may be prevented by the addition of the appropriate cofactors in excess to the reaction mixture. Such a cofactor supplement is most effective in countering the steroid inhibition if incubated with enzyme preparation and substrate before hydrocortisone is added.

Loss of respiratory cofactors is probably secondary to the effect of hydrocortisone on permeability of mitochondrial membranes. Selective semi-permeability is lost rapidly and extensively on the addition of the steroid to intact mitochondria. When permeability barriers are removed by suspension of mitochondria in water, hydrocortisone has no effect on the activity of L-malic dehydrogenase.

The mechanism of inhibition is thus probably a direct effect of hydrocortisone on the mitochondrial membrane. Permeability is increased rapidly and greatly, leading to the loss of soluble respiratory cofactors, particularly the pyridine nucleotides, from mitochondrial enzyme systems. The activities of pyridine nucleotide-dependent dehydrogenases are secondarily inhibited by lack of their electron acceptor. It seems possible that the same mechanism may be operative for other steroids.

Evidence has been published which favours a mechanism *in vivo* similar to the mode of action defined in this study. Eisenberg, Gordan & Elliott (1949*a*, *b*) have shown that castration of rats leads to a significant increase in brain respiration, which is reduced to almost normal rates by the administration of testosterone. On the other hand, liver and muscle respiration is not affected by castration, indicating a tissue specificity which may reflect higher sensitivity of brain or increased local concentrations of the steroid in this tissue. Clark & Pesch (1956) found that the administration of cortisone to rats led, among other things, to inhibition of α-oxoglutarate oxidation by mitochondria without lowering the efficiency of oxidative phosphorylation with this substrate. Similarly Hennes, Wajchenberg, Fajans & Conn (1957) found evidence for a block in pyruvate utilization after the administration of adrenal steroids to

normal humans, and Lacroix & Leusen (1958) found that the daily administration of cortisone to rats for 12–17 days considerably depressed the tissue-slice respiration of myocardium.

Such evidence confirms that steroids can inhibit oxidative metabolism *in vivo*. It remains to be shown that the same mechanism is operative *in vivo* and *in vitro*. Hartman (1956) found that mitochondria of the central nervous system became vacuolated after administration of cortisone to rats. This may be explained by an effect of the steroid on the mitochondrial membranes, leading to the loss of selective permeability and rendering them freely permeable. It is possible that, at least in the central nervous system, cortisone influences oxidative metabolism in the same way *in vivo* as hydrocortisone exerts its effect *in vitro*, by increasing the permeability of mitochondrial membranes. Such a mechanism indeed forms an attractive hypothesis for the manner in which adrenal steroids, and probably steroids generally, regulate metabolism.

SUMMARY

1. Hydrocortisone has been shown to inhibit oxidative metabolism *in vitro* by liver mitochondria. Oxidative phosphorylation is not affected.

2. The inhibitory effect is specific to dehydrogenases requiring pyridine nucleotides for electron transfer.

3. The steroid does not inhibit L-malic dehydrogenase directly.

4. Inhibition may be prevented by supplementing the reaction mixture with excess of pyridine nucleotide, nicotinamide, glutathione and, for octanoate oxidation, coenzyme A. The protective effect is greatest if the cofactor supplement is incubated with enzyme and substrate before hydrocortisone is added.

5. Hydrocortisone, in concentrations which produce oxidative inhibition, has been shown to increase the permeability of mitochondrial membranes. Elimination of permeability barriers by suspension of mitochondria in water, instead of in 0·25 M-sucrose, removes completely the effect of hydrocortisone on L-malic dehydrogenase activity.

6. Hydrocortisone is considered to inhibit oxidative metabolism by the following mechanism. The primary effect is to increase the permeability of mitochondrial membranes, allowing loss of soluble respiratory cofactors from mitochondrial-enzyme systems. Dehydrogenases dependent on pyridine nucleotides for electron transfer are thus proportionally inactivated.

7. The applicability of findings *in vitro* to the reported effects *in vivo* of steroids is discussed.

8. Hydrocortisone is postulated as one of the regulatory factors governing selective semi-permeability of mitochondrial membranes *in vivo* and thereby of tissue metabolism.

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Condensed Tannins

3. ISOLATION AND ESTIMATION OF (−)-7:3′:4′:5′-TETRAHYDROXYFLAVAN-3-OL, (+)-CATECHIN AND (+)-GALLOCATECHIN FROM BLACK-WATTLE-BARK EXTRACT*

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The bark and heartwood extractives of the black-wattle tree (*Acacia mollissima*) both contain mixtures of complex polyphenolic compounds consisting of a molecular-weight gradation within the range 300–3000 (Roux & Evelyn, 1958*a*, *c*). Keppler (1957) showed that wattle-heartwood extractives contain a (+)-7:3′:4′-trihydroxyflavan-3:4-diol, which appears to be the prototype and possible precursor of some of the condensed tannins of the wood (Roux, 1958*b*), and which was found to be enantiomorphous (Clark-Lewis & Roux, 1958, 1959) with a similar monomeric (−)-leuco-fisetinidin prototype of quebracho (*Schinopsis balansae* and *S. quebracho-colorado*) heartwood tannins (Roux, 1958*a*; Freudenberg & Weinges, 1958; Roux & Evelyn, 1958*b*). Roux & Maihs (1958) have briefly reported the isolation from the fresh bark extract of (−)-7:3′:4′:5′-tetrahydroxyflavan-3-ol, in addition to (+)-catechin and (+)-gallocatechin. The present study provides details of the isolation, characterization and estimation of these catechins.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Mixed melting points were made on the crystalline residue remaining after dissolving equal weights of both compounds in methanol and removing the methanol under vacuum. Analyses of C, H, methoxyl and acetyl are by Weiler and Strauss, Oxford. Infrared comparisons were made by Dr J. R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria. Tannin analyses are by the ultraviolet photometric method for black-wattle tannins (Roux, 1951, 1957*a*, *b*), standardized against the hide-powder (shake) method.

Isolation and identification of (−)-7:3′:4′:5′-*tetrahydroxyflavan-3-ol* [(−)-*robinetinidol*], (+)-*catechin and* (+)-*gallocatechin*

Preliminary fractionation of bark extracts. The damp, mature (5–10 mm. thick) and freshly stripped bark from *A. mollissima* was sliced as soon as possible into slivers of 1 mm. thickness by cutting across the grain with a stainless-steel knife, and the slivers were immediately covered with commercial ethyl acetate. Extraction at room temperature proceeded over 5 days with daily renewal of the ethyl acetate. The combined extracts were evaporated to dryness in a rotary evaporator under vacuum, yielding a pale cream-coloured product. This ethyl acetate-soluble extract contained 92·5% of tannins, and the tannin content of the whole extract represented 62% of the total content of the bark. A methanol-soluble extract obtained by the same method contained 70% of tannins, representing 96·5% of the total bark tannins. The ethyl acetate-soluble fraction is of lower (921) average molecular weight (Evelyn, 1958) compared with that of the whole fresh-bark extract (1275) but still contains much high-molecular-weight tannins. The latter are removed by one of two alternative procedures: *Procedure* 1. The ethyl acetate-soluble tannins (10 g.) are dissolved in ethyl acetate (100 ml.), and $CHCl_3$ (100 ml.) is added in small quantities with vigorous shaking. The white precipitate formed was filtered from the straw-coloured liquid under anhydrous conditions, and the latter evaporated to dryness under vacuum (0·9 g.). *Procedure* 2. A similar product was obtained by chromatography on a Solka Floc cellulose (Brown Co., Berlin, New Hampshire, U.S.A.) column (4 cm. × 55 cm.), prepared by introducing the cellulose in a slurry with water and washing with 2% acetic acid (8 hr.) and water (8 hr.). The ethyl acetate-soluble tannins (25 g.) in water (125 ml.) were introduced and the column was developed with water. After the emergence of the first polyphenols in the eluent ($FeCl_3$ test), 625 ml. was collected in one fraction over 8 hr. From this fraction 16 g. of extract was recovered by evaporation under vacuum.

Separation of catechins by paper chromatography. The products from separation procedures 1 and 2 were streaked (400 mg. per sheet) on to a Whatman no. 3 (18½ in. × 22½ in.) paper sheet and the chromatogram was developed in 2% acetic acid by upward migration. The air-dried sheets were examined under ultraviolet light. Fluorescent bands were observed at R_F values 0·43 (dark-blue A band), 0·48 (dark-blue B band), 0·56 (dark-blue C band), 0·64 (light-blue E band) and 0·73 (dark-mauve H band). Bands B and C were separated by a very narrow bright-blue fluorescent band which serves as an excellent marker and enabled these bands to be demarcated accurately.

Strips were cut corresponding to bands B and C, and these were eluted with 70% ethanol (Nordström & Swain, 1953*a*). Band C eluate was evaporated under vacuum until the ethanol was removed. Microcrystals separated on standing. Yield: 2·4 g. from 720 g. of ethyl acetate extract enriched by procedure 1, and 0·25 g. from 102 g. of ethyl acetate extract enriched by procedure 2. Band B eluates were evaporated to dryness under vacuum (yield 13–15%

* Part 2. Roux & Evelyn (1958*b*).

of enriched fraction), and streaked on to washed (Nordström & Swain, 1953*a*) Whatman no. 3 chromatographic sheets (200 mg. per sheet). The chromatograms were developed in butan-1-ol–acetic acid–water (6 : 1 : 2) and examined under ultraviolet light. Two dark-mauve bands B_1 (R_F 0·70) and B_3 (R_F 0·53) were visible and separated by a light-blue band B_2 (R_F 0·64). Bands B_1 and B_2 were cut from the sheets and eluted as before. After removal of ethanol and concentration under vacuum, crystals separated from each of the aqueous concentrates.

(−)-7:3′:4′:5′-*Tetrahydroxyflavan*-3-*ol*. The crystals from band C consisted only of component H (see Roux, 1953; Roux & Evelyn, 1958*a*) having R_F values 0·76 (butan-1-ol–acetic acid–water, 4 : 1 : 5), 0·67 (water-saturated butan-2-ol) and 0·47 in 2% acetic acid. Component H crystallized from water as a white hydrate, m.p. 207°, $\lambda_{max.}$ 282 mμ (Found: C, 56·2; H, 5·8; loss at 100°, 8·8. Calc. for $C_{15}H_{14}O_6$,1·5H_2O: C, 56·8; H, 5·4; H_2O, 8·5%). After drying over P_2O_5 at room temperature or at 80° under vacuum, one molecule of water was retained (Found: C, 58·6; H, 5·4. Calc. for $C_{15}H_{14}O_6$,H_2O: C, 58·4; H, 5·2%), but this was lost by drying at 100° for 2 hr. (Found: C, 61·7; H, 5·1. Calc. for $C_{15}H_{14}O_6$: C, 62·1; H, 4·9%). A blue with $FeCl_3$ indicated the presence of a pyrogallol unit; a very weak reaction with vanillin–toluene-*p*-sulphonic acid reagent (Roberts, Cartwright & Wood, 1956) indicated the absence of phloroglucinol nucleus, and a yellow with bisdiazotized benzidine (Lindstedt, 1950) indicated the probable presence of a resorcinol nucleus.

Fusion with KOH under anhydrous conditions resulted in degradation to β-resorcylic acid (also some resorcinol) and gallic acid. In the partitioning mixtures water-saturated butan-2-ol and butan-1-ol–acetic acid–water it migrated at the same rate as (+)-catechin and (+)- and (−)-7:3′:4′-trihydroxyflavan-3:4-diol, suggesting (1) the presence of a total of five hydroxy groups on the C_{15} skeleton, and (2) that the compound was a catechin containing pyrogallol and resorcinol nuclei, e.g. 7:3′:4′:5′-tetrahydroxyflavan-3-ol. Its melting point and specific rotation, $[\alpha]_D^{20}$ −10·7±0·3° in acetone–water (1 : 1) (*c*, 1·42), suggested its identity with (−)-robinetinidol, m.p. 207–209°, $[\alpha]_D^{25}$ −11·5°, obtained shortly before by Weinges (personal communication and 1958) from the catalytic reduction of (+)-dihydrorobinetin by the method of Freudenberg & Roux (1954) (see also Roux & Freudenberg, 1958) followed by the catalytic hydrogenolysis of the resulting (+)-7:3′:4′:5′-tetrahydroxyflavan-3:4-diol at position 4.

Component H from wattle does not assume a pink on drastic drying as does synthetic (−)-robinetinidol (Weinges, 1958), but a mixed m.p. of the natural and synthetic compounds showed almost no depression (m.p. 204°), and they also show superimposable infrared-absorption curves over the range 2·5–15 μ. On two-dimensional chromatograms with water-saturated butan-2-ol for direction 1 and 2% acetic acid for direction 2, the natural and synthetic compounds run to identical positions.

7:3′:4′:5′-*Tetramethoxyflavan*-3-*ol*. (−)-7:3′:4′:5′-Tetrahydroxyflavan-3-ol (500 mg.) from wattle-bark extract was methylated with diazomethane by the method described for (−)-leuco-fisetinidin (Roux & Evelyn, 1958*b*), the reaction mixture being kept at −5° for 2 days in the presence of excess of diazomethane. After concentration of solvents the product was poured into water and the white flocculent precipitate recrystallized from ethanol (0·41 g.). The melting point, 141–142° increasing to 143–144° after two further recrystallizations, was similar to that of synthetic 7:3′:4′:5′-tetramethoxy-(−)-robinetinidol (Weinges, 1958) and a mixed m.p. of the natural and synthetic derivatives showed no depression (Found: C, 65·9; H, 6·7; OMe, 35·4. Calc. for $C_{19}H_{22}O_6$: C, 65·9; H, 6·4; OMe, 35·8%). The specific rotation, $[\alpha]_D^{20}$ −21·6±0·4° in tetrachloroethane (*c*, 2·3), was also similar to that of the synthetic derivative (−22·3°) and their infrared-absorption curves were identical over the range 2·5–15 μ.

3:7:3′:4′:5′-*Penta-acetylflavan*. Component H (490 mg.) was acetylated with acetic anhydride (5 ml.) and sodium acetate (1 g.) as described (Roux & Evelyn, 1958*b*) for 3:4-diacetoxy-7:3′:4′-trimethoxyflavan. The solid obtained (0·6 g.) was repeatedly recrystallized from ethanol to m.p. 132–133°, $[\alpha]_D^{20}$ +4·6±0·3° in tetrachloroethane (*c*, 2·15) (Found: C, 60·0; H, 4·9; $CO.CH_3$, 42·0. $C_{25}H_{24}O_{11}$ requires C, 60·0; H, 4·8; $CO.CH_3$, 43·0%). This derivative was not reported by Weinges (1958) but (personal communication) was prepared by him, m.p. 131–133°, $[\alpha]_D^{25}$ +4·9° in tetrachloroethane (*c*, 2). Mixed melting points of the synthetic and natural derivatives showed no depression, and their infrared-absorption curves were superimposable over the range 2·5–15 μ.

(+)-*Catechin*. The crystals from band B_1 correspond to extract component G (0·83 g. from 720 g. of ethyl acetate-soluble tannins with separation procedure 1), and were recrystallized from water, m.p. 172–174°, $[\alpha]_D^{21}$ +17·0° in acetone–water (1 : 1) (*c*, 1·1), $\lambda_{max.}$ 280 mμ (Found: C, 56·8; H, 5·7. Calc. for $C_{15}H_{14}O_6$,1·5H_2O: C, 56·8; H, 5·4%). A mixed melting point with (+)-catechin from gambier extract from the leaves of *Uncaria gambir* showed no depression, and the infrared-absorption curves of (+)-catechin and component G were superimposable over the range 2·5–15 μ. With $FeCl_3$ (green), bisdiazotized benzidine (ochre) and vanillin–toluene-*p*-sulphonic acid (violet) reagents, constituent G gave the same colour reactions as (+)-catechin and migrated to identical positions in two-way solvent systems which separate optical antipodes (Roberts & Wood, 1953). R_F values in water (0·35), water-saturated butan-2-ol (0·67) and butan-1-ol–acetic acid–water (4 : 1 : 5) (0·75) are in agreement with those of (+)-catechin (Bradfield & Bate-Smith, 1950; Roux & Evelyn, 1958*c*). The penta-acetyl derivative of component G, m.p. 131–132°, $[\alpha]_D^{20}$ +40·1° in tetrachloroethane (*c*, 1·3), was formed as above for 3:7:3′:4′:5′-penta-acetylflavan. Mixed m.p. with an authentic specimen of penta-acetyl-(+)-catechin showed no depression.

(+)-*Gallocatechin*. The microcrystals from band B_3, composed mainly of component A_2, were recrystallized from water (30 mg. from 720 g. of ethyl acetate-soluble tannins with procedure 1), m.p. and mixed m.p. with (+)-gallocatechin from *Casuarina equisetifolia* (Roux, 1957*c*) 182–184°, $[\alpha]_D^{20}$ +10·7° in acetone–water (1 : 1) (*c*, 0·5), $\lambda_{max.}$ 271 mμ. The crystals tended to darken rapidly on drying (Found: C, 55·8; H, 5·4. Calc. for $C_{15}H_{14}O_7$,H_2O: C, 55·6; H, 5·0%). Infrared-absorption curves of component A_2 and (+)-gallocatechin were identical over the range 2·5–15 μ.

Component A_2 developed the same coloration with $FeCl_3$ (blue), bisdiazotized benzidine (ochre) and vanillin–toluene-*p*-sulphonic acid (violet) reagents as with (+)-gallocatechin, and migrated to identical positions on two-way

chromatograms under conditions which allow for the separation of optical antipodes (Roberts & Wood, 1953). R_F values of A_2 in water (0·32), butan-1-ol–acetic acid–water (4 : 1 : 5) (0·57) and water-saturated butan-2-ol (0·54) are in agreement with those of (+)-gallocatechin (Bradfield & Bate-Smith, 1950; Roux & Evelyn, 1958*c*).

Examination of wattle extract for the presence of (−)-*epicatechin*. The catechin-enriched fractions of wattle-bark extracts, bands B and C, and also band A of lower R_F in 2% acetic acid, were examined by two-way chromatography and spray reagents as described above for the presence of (+)- or (−)-*epi*catechin, with (−)-*epi*catechin from cocoa beans (Forsyth, 1954) as reference compound. With reference to the work of Roberts & Wood (1953), the apparent absence of *epi*catechins in fresh bark extracts was established, and no significant quantities of (−)-*epi*catechin could be detected in the commercial extract.

Estimation of catechins in black-wattle extracts

The percentages of (−)-7:3′:4′:5′-tetrahydroxyflavan-3-ol, (+)-catechin and (+)-gallocatechin in methanol and ethyl acetate extractives of fresh bark were determined by the measurement of the colour density of their spots on two-way chromatograms with a simple densitometer (Block, 1950; Block, Durrum & Zweig, 1955) procedure previously used for amino acids. All chromatograms were run by the ascending method, at 21° in water-saturated butan-2-ol in the short direction (11¼ in.; 15 hr.) and, after drying, in the long direction (18¼ in.; 5–6 hr.).

The catechins dissolved in methanol (1% solution) were spotted on the sheets in 10 μg. increments over the range 10–100 μg., and the extracts in 0·5 mg. increments (10% solution) over the range 1–8 mg. The dried chromatograms were sprayed with one of the following: (*a*) ammoniacal $AgNO_3$ reagent containing 14 g. of $AgNO_3$/100 ml. Chromatograms were sprayed with this freshly made reagent until damp, left 2–3 min. for full development of brown colour and finally washed in a dish, first with water (three thorough washings) followed by treatment with 0·5% sodium thiosulphate solution for 2 min. and washing in running water for 30 min. (*b*) Benzidine reagent (Lindstedt, 1950), consisting of a solution of 5 g. of benzidine, 14 ml. of conc. HCl and 980 ml. of water freshly mixed with two-thirds of its volume of aqueous 10% sodium nitrite solution. After spraying until the maximum colour density had developed, the chromatograms were washed in running water for 30 min. to remove excess of benzidine reagent.

Densitometer and method of measurement. The maximum colour density of the spots, after spraying, was measured by using a densitometer and method similar to that described by Block *et al.* (1955). The colour density of each spot was determined by using the blank paper of the particular chromatogram as 100% transmission. A straight-line relationship between log concentration and galvanometer readings was obtained for each catechin. From these standard curves an approximate estimation of catechins in the methanol and ethyl acetate-soluble extracts of the fresh bark was made (Table 1). (+)-Catechin and (−)-robinetinidol run well in advance of other components on two-way paper chromatograms (components G and H respectively, see Roux & Evelyn, 1958*b*), but gallocatechin (component A_2) is closely associated with others in the A complex, and also with B and F, and its estimation is complicated by the close proximity of these reducing bodies when the $AgNO_3$ spray reagent is used. With the benzidine reagent, however, the ochre coloration of (+)-gallocatechin permits the more accurate estimation, both visual and photometric, of this compound in the presence of other bodies which furnish yellow colorations. The value obtained for (+)-catechin with the benzidine reagent (1·2%) was very similar to that obtained with the $AgNO_3$ spray (1·4%), whereas that for (+)-gallocatechin with the benzidine reagent was half (1·8%) the value obtained with the $AgNO_3$ spray (3·6%).

Table 1. *Estimation of catechins in black-wattle extract*

Values are expressed as percentage of dry extract and, in parentheses, as percentage of the tannin content.

Bark extract	(−)-Robinetinidol	(+)-Catechin	(+)-Gallocatechin
	$AgNO_3$ spray		
Methanol	0·7 (1·0)	1·1 (1·6)	2·8* (4·0)*
Ethyl acetate	0·8 (0·9)	1·4 (1·5)	3·6* (3·9)*
	Benzidine spray		
Ethyl acetate	—	1·2 (1·3)	1·8 (1·9)
	By isolation		
Ethyl acetate	0·33	0·12	0·0004

* Values erroneously high due to interference or overlap of other polyphenols. See text.

DISCUSSION

Black-wattle-bark extracts contain polyphenolic and carbohydrate fractions in the approximate proportion of 3 : 1. The carbohydrate fraction consists partly of a mixture of sucrose, glucose and fructose (paper chromatography), as well as about 7% of the total extract weight of hydrocolloid gums (Roux, 1952). From the polyphenolic fraction, comprising 60% of the commercial bark extract, fisetin, gallic acid and resorcinol (Roux, 1952), and syringic acid (King, 1956), have been isolated in exceptionally low yield. Roux (1953) provisionally identified (+)-catechin and (+)-gallocatechin by paper chromatography from amongst the main polyphenolic components of the fresh-bark extract. King (1957) isolated milligram quantities of (+)-catechin (m.p. 173–174°) and an impure substance (m.p. 166°) which was surmised to be a 3:7:3′:4′:5′-pentahydroxyflavan on the basis of degradative and quantitative colorimetric evidence only. *epi*Catechin (rotation not designated) was also shown to be present by paper chromatography, but King could not establish the presence of gallocatechin by this method. The above-described and previous work on wattle extract has emphasized the difficulty experienced in isolating crystalline components from the polyphenolic fraction. The presence of a high percentage of tannins varying in average molecular weight over the range 600–3000 (Roux & Evelyn, 1958*a*, *c*) appears to complicate separation procedures.

In the present work (−)-7:3′:4′:5′-tetrahydroxyflavan-3-ol, (+)-catechin and (+)-gallocatechin have been isolated in sufficient quantity for their accurate identification. (−)-*epi*Catechin appears to be absent from both fresh-bark and commercial extracts (compare King, 1957). The high melting point (207°) of (−)-7:3′:4′:5′-tetrahydroxyflavan-3-ol [(−)-robinetinidol] emphasizes the relative impurity (m.p. 166°) of that substance, which King (1957) correctly surmised to be a 3:7:3′:4′:5′-pentahydroxyflavan. The shift in the specific rotation of penta-acetyl-(−)-robinetinidol relative to that of the parent compound (positive), to the tetramethyl ether (positive), pentamethyl ether (positive), tetramethyl-3-acetyl (positive) and tetramethyl-3-tosyl (negative) derivatives bears the same relationship as penta-acetyl-(+)-catechin to the corresponding (+)-catechin derivatives (see Weinges, 1958). The shifts relative to the tetramethyl-3-mesyl derivatives constitute an anomaly. This evidence (also communicated by Dr Weinges) nevertheless confirms his deduction of the agreement in absolute configuration between (+)-catechin and (−)-robinetinidol. From the interrelationships between the catechins, leuco-anthocyanidins and 2:3-dihydroflavonols (Weinges, 1958; Clark-Lewis & Roux, 1958) it follows that the flavans of the resorcinol series present in *A. mollissima*, namely (−)-robinetinidol from the bark and (+)-mollisacacidin from the wood, have the same (2:3-*trans*) configurations in C atoms 2 and 3.

The concentration of (+)-catechin in the methanol or ethyl acetate extracts of the fresh bark (1·1–1·4 %) is higher than that found by King & White (1956) for the commercial extract (0·02 %).

Table 2. *Distribution of* C_{15} *'flavonoid' compounds of the resorcinol series in Nature*

Compound	Species	Reference
Family: Leguminosae		
Catechins or flavan-3-ols		
(−)-7:3′:4′:5′-Tetrahydroxy- (robinetinidol)	*Acacia mollissima*	Roux & Maihs, 1958
Flavan-3:4-diols		
(+)-7:3′:4′:5′-Tetrahydroxy-	*Robinia pseudacacia*	Weinges, 1958
(+)-7:3′:4′-Trihydroxy- (mollisacacidin, gleditsin)	*A. mollissima* *Gleditsia japonica*	Keppler, 1957 Mitsuno & Yoshizaki, 1957
7:4′-Dihydroxy-	*A. pycnantha*, *Guibourtia* spp. *Guibourtia* spp.	Roux, 1959
2:3-*Dihydroflavonols*		
7:3′:4′:5′-Tetrahydroxy-	*R. pseudacacia*	Freudenberg & Hartmann, 1954
7:3′:4′-Trihydroxy- (fustin)	*Gleditsia triacanthos* *G. japonica*	Chadenson, Molho-Lacroix, Molho & Mentzer, 1955
Flavonols		
7:3′:4′:5′-Tetrahydroxy- (robinetin)	*R. pseudacacia* *Gleditsia monosperma*	Schmidt & Pietsch, 1931
7:3′:4′-Trihydroxy- (fisetin)	*G. japonica*	Mitsuno & Yoshizaki, 1957
2:3-*Dihydroflavones*		
7:3′:4′-Trihydroxy- (butin)	*Butea frondosa*	Perkin & Hummel, 1904
7:4′-Dihydroxy- (liquiritigenin)	*Glycyrrhiza glabra*	Shinoda & Ueeda, 1934
iso*Flavones*		
7-Hydroxy-4′-methoxy-	*Trifolium subterraneum* *T. pratense*	Bradbury & White, 1951
Family: Anacardiaceae		
Flavan-3-4-diols		
(−)-7:3′:4′-Trihydroxy- [(−)-leuco-fisetinidin]	*Schinopsis quebracho-colorado*	Roux, 1958*a*; Freudenberg & Weinges 1958
2:3-*Dihydroflavonols*		
7:3′:4′-Trihydroxy- (fustin)	*Rhus succedanea* *R. cotinus*, *R. glabra*, *R. vernicifera*, *R. trichocarpa*	Oyamada, 1934
Flavonols		
7:3′:4′:5′-Tetrahydroxy-	*Schinopsis* spp.	Kirby & White, 1955
7:3′:4′-Trihydroxy- (fisetin)	*S. quebracho-colorado* *Rhus* spp. (see above)	Perkin & Gunnell, 1896
7:3′-Dihydroxy-4′-methoxy-	*Schinopsis* spp.	Kirby & White, 1955
7:4′-Dihydroxy-	*Schinopsis* spp.	Kirby & White, 1955

The complex condensed tannins of black-wattle bark show light-absorption and degradative behaviour typical of the flavans, and the catechin, 3:7:3′:4′:5′-pentahydroxyflavan (robinetinidol) has for many years been regarded as a possible prototype of wattle tannins (Roux & Maihs, 1958). No direct chemical or biochemical evidence as for the leuco-fisetinidins of quebracho tannins (Roux, 1958*b*; Roux & Evelyn, 1958*b*) has, however, been obtained to support this surmise. The work of Hathway (1959) on oak-bark tannins suggests that pyrogallol catechins in addition to leuco-anthocyanidins, participate in condensed tannin formation.

The simultaneous presence of (+)-catechin and (+)-gallocatechin in wattle bark is of interest in view of their parallel occurrence in the bark tannins of *Casuarina equisetifolia* (Roux, 1957*c*), *Quercus sessifolia*, *Castanea vesca* (Mayer & Bauni, 1956, 1957) and *Quercus pendunculata* (Hathway, 1959).

Similarly, the presence of (−)-robinetinidol [and also of (+)-mollisacacidin] in *Acacia mollissima* lends further emphasis to the almost exclusive occurrence amongst the Leguminosae and two genera of the Anacardiaceae (namely *Rhus* and *Schinopsis*), of all those C_{15} 'flavonoid' compounds of the resorcinol series which have hitherto been isolated and identified in Nature (Table 2). The only known exceptions amongst a broader group of C_{15} compounds are the resorcinol-containing chalcones, butein and dahlia chalcone, and benzilidenecoumaranones (aurones), for example sulphuretin, from the influorescence of certain Compositae (see Nordström & Swain, 1953*b*; Bate-Smith, Swain & Pope, 1953). This rather remarkable distribution, if also confirmed by future investigation, may be of taxonomic significance, and provides confirmation of plant classifications proposed on the basis of morphological and anatomical characteristics similar to that found by Erdtman (1955) for the Coniferae.

SUMMARY

1. (−)-7:3′:4′:5′-Tetrahydroxyflavan-3-ol [(−)-robinetinidol] has been isolated from black-wattle-bark (*Acacia mollissima*) extracts in 0·3 % yield.

2. (+)-Catechin and (+)-gallocatechin were isolated in lower yields from the extracts, and (−)-*epi*catechin has been shown to be absent from fresh-bark and commercial extracts.

3. The concentration of the catechins in the fresh-bark extracts and in their tannin fractions is estimated by means of paper chromatography in conjunction with the measurement of maximum colour density of the spots with a densitometer.

4. The presence of (−)-robinetinidol in *Acacia mollissima* places further emphasis on the almost exclusive occurrence amongst the Leguminosae and two genera of the Anacardiaceae of those C_{15} 'flavonoid' compounds of the resorcinol series which have hitherto been isolated.

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The Enzymic Synthesis of Aryl Sulphamates

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Boyland, Manson & Orr (1957) have shown that the administration of certain arylamines to rats or to rabbits is followed by the urinary excretion of the corresponding aryl sulphamates, compounds whose natural occurrence had not previously been recognized. This was followed by the demonstration of the synthesis *in vitro* of 2-naphthyl sulphamate by rat-liver preparations (Roy, 1958) and of phenyl sulphamate by sheep intestinal-mucosal enzymes (Kent & Pasternak, 1958). These results were of interest, as the reactions involved might be expected to be related to those involved in the synthesis of the sulphamate groups of heparin (Foster & Huggard, 1955). In the light of the present ideas on the synthesis of sulphuric acid esters (Lipmann, 1958) it seemed likely that the synthesis of aryl sulphamates would involve sulphate transfer from 'active sulphate', adenosine 3′-phosphate 5′-sulphatophosphate, this reaction being catalysed by a specific sulphokinase, arylamine sulphokinase. The present paper describes an investigation into the enzymic synthesis of aryl sulphamates and, in particular, into the influence of certain steroids on this reaction. A preliminary account of the work has already appeared (Roy, 1959).

EXPERIMENTAL

Preparation of the enzyme system. This was carried out essentially as previously described (Roy, 1956). With 5 g. samples of liver the tissue was treated with 3 vol. of 0·15 M-KCl containing mM-ethylenediaminetetra-acetate (EDTA), pH 7, in a glass homogenizer, for two 60 sec. periods. Larger amounts of tissue were treated with 3 vol. of the KCl solution in an Atomix blender (M.S.E. Ltd.) for two 30 sec. periods. The resulting tissue suspension was centrifuged for 2 hr. at 20 000 *g* and the supernatant precipitated between 1·5 and 2·3 M-$(NH_4)_2SO_4$, to give the enzymically active fraction. This was dissolved in water so that 1 ml. of the resulting solution corresponded to 500 mg. of liver; the protein concentration was approximately 18 mg./ml.

The sulphate-activating system, a mixture of adenosine triphosphate (ATP) sulphurylase and adenosine 5′-sulphatophosphate phosphokinase (Lipmann, 1958), was prepared by heating the above enzyme solution at 52° (DeMeio, Wizerkaniuk & Schreibman, 1955) for 30 min. and removing the denatured protein by centrifuging.

The sulphate-activating system from baker's yeast was prepared exactly as described by Nose & Lipmann (1958).

The sulphokinase fraction of the enzyme was prepared from the complete enzyme system by dialysing the latter for 48 hr. against repeated changes of 100 vol. of water at 0°, a procedure which destroyed the sulphate-activating system because of the latter's instability in the absence of SO_4^{2-} ions. The resulting suspension was made 0·03 M in glycylglycine and adjusted to pH 8 with N-Na_2CO_3. After the suspension had stood for a few hours any remaining insoluble material was removed by centrifuging. The enzyme suspension obtained after dialysis could be used without further treatment, but a considerable proportion of the sulphokinase was present in the insoluble fraction and was rather unstable in this state, the activity being destroyed on freezing. The soluble sulphokinase preparations obtained after glycylglycine treatment were stable and could be stored frozen for long periods.

Preparation of adenosine 3′-phosphate 5′-sulphatophosphate. This was prepared enzymically by incubating 100 ml. of a mixture containing 50 ml. of the above unfractionated enzyme preparation, 10 ml. of 0·2 M-phosphate buffer (pH 7·2), 10 ml. of 0·04 M-$MgSO_4$–0·06 M-K_2SO_4 and 400 μmoles of ATP (crystalline sodium salt, Sigma Chemical Co., adjusted to pH 7·2 with N-NaOH) for 1 hr. at 37°.

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Proteins were precipitated by adding 3 vol. of ethanol and removed by centrifuging. The ethanol was removed from the supernatant by concentrating it *in vacuo* at a bath temperature of 40° to about 25 ml.; the resulting solution was brought to pH 8 with N-NaOH, insoluble material removed by centrifuging and the volume made up to 50 ml. with water. The resulting solution was stored frozen at −10°.

This preparation contained nucleotides other than adenosine 3′-phosphate 5′-sulphatophosphate, but was used for experiments involving assays of the sulphokinase fraction, prepared as above, which cannot utilize ATP and SO_4^{2-} ions for the synthesis of sulphate esters.

Chromatography. Chromatographic separation of the metabolites of 2-naphthylamine, and related arylamines, was carried out by descending chromatography on Whatman no. 1 paper with the solvent system butan-1-ol–propan-1-ol–aq. 0·1 N-NH_3 (2:1:1, by vol.) as described by Boyland *et al.* (1957). 2-Naphthyl sulphamate and other arylamine metabolites containing a free, or potentially free, amino group were detected as orange–yellow spots after spraying successively with N-HCl, 0·1% (w/v) $NaNO_2$ and 0·5% (w/v) thymol in 2N-NaOH. They could also be detected by spraying with Ehrlich's reagent [0·5% (w/v) dimethylaminobenzaldehyde in 0·1 N-HCl in ethanol] and heating at 37° for 30 min. The R_F values of 2-naphthyl sulphamate and 2-naphthylamine were 0·26 and 0·83 respectively.

Determination of 2-naphthyl sulphamate. This was carried out by hydrolysis to 2-naphthylamine, followed by diazotization and coupling. To 3 ml. of sulphamate solution, free from naphthylamine, was added 0·3 ml. of conc. HCl and the mixture heated for 30 min. in boiling water. After cooling, 1·0 ml. of 0·1% (w/v) $NaNO_2$ was added, followed after 5 min. by 5 ml. of 0·5% (w/v) thymol in 5N-NaOH. The intensity of the resulting orange–yellow colour was read in the Spekker absorptiometer, with Ilford filter 602 (maximum transmission at 470 mμ). Calibration curves prepared from 2-naphthyl sulphamate and 2-naphthylamine were identical.

The method could be applied to the determination of other aryl sulphamates, although with some, such as phenyl sulphamate, acid hydrolysis was not required, as the sulphamate decomposed quantitatively during the diazotization.

Assay of 2-naphthyl sulphamate synthesis. The method varied, according to the object of the assay and the enzyme preparation used. With the unfractionated enzyme system the volume of the reaction mixture was 5 ml., containing 0·5 ml. of buffer (see below), 0·5 ml. of 0·04 M-$MgSO_4$–0·06 M-K_2SO_4, 1 ml. of 0·02 M-ATP (crystalline sodium salt, adjusted to the appropriate pH with N-NaOH), 1 ml. of 0·01 M-2-naphthylamine hydrochloride and 2 ml. of enzyme solution. With enzyme preparations from guinea-pig liver the amount used corresponded to approximately 60 mg. of tissue, but with rat preparations some 200 mg. of tissue was required. Incubation was for 2 hr. at 37°. Control assays were simultaneously carried out under identical conditions except that no ATP was added. Values obtained by these controls were identical with those in which the enzyme was incubated separately from the remainder of the reaction mixture (containing ATP).

For the qualitative demonstration of sulphamate synthesis by chromatography the buffer used was 0·2 M-potassium phosphate buffer, pH 7·2: this buffer was chosen despite the fact that the pH was not optimum because it was to a large extent eliminated by the subsequent treatment and so did not interfere with the chromatography. The reaction was stopped by the addition of 25 ml. of ethanol, the precipitate removed by centrifuging and the supernatant evaporated to dryness *in vacuo*. The residue was taken up in 0·3 ml. of water, insoluble material centrifuged off and 0·02 ml. samples were used for chromatography as described above.

Quantitative assays were carried out as above but with 0·5 M-2-amino-2-hydroxymethylpropane-1:3-diol–acetic acid buffer (tris buffer), pH 8·2, and the mixture was deproteinized by the addition of 2 ml. of 25% (w/v) trichloroacetic acid. Proteins were centrifuged off and free amino compounds were removed from the supernatant by shaking it for two 15 min. periods, each with 2 g. of Zeo-Karb 225 H^+ (Permutit Co. Ltd., London). After centrifuging, 3 ml. samples of the resulting solution were taken for the determination of sulphamate as described above.

The technique used in the assay of sulphamate synthesis from adenosine 3′-phosphate 5′-sulphatophosphate by sulphokinase preparations was similar. The volume of the reaction mixture was 5 ml., containing 1 ml. 0·5 M-tris buffer (pH 8·2), 1 ml. of 0·01 M-magnesium acetate, 1 ml. of 0·01 M-2-naphthylamine hydrochloride, 1 ml. of the adenosine 3′-phosphate 5′-sulphatophosphate solution described above and 1 ml. of a sulphokinase preparation corresponding to some 250 mg. of liver. Incubation was at 37°, for 1 hr. with guinea-pig preparations, but for 2 hr. with rat preparations. The reaction was stopped by the addition of 2 ml. of 25% (w/v) trichloroacetic acid and thereafter the procedure was as described above for the assay with unfractionated enzyme preparations. Controls in which adenosine 3′-phosphate 5′-sulphatophosphate was omitted from the reaction mixture were carried out with each set of determinations.

Assay of steroid and aryl sulphate synthesis. This was carried out as previously described by using the methylene-blue technique (Roy, 1956). The ATP concentration used in these assays was 4 mM, not 2 mM as originally described: this higher optimum ATP concentration was apparently due to the use of crystalline ATP preparations in the present series of determinations. The substrate concentrations used were: *p*-nitrophenol, 1 mM; oestrone, 0·1 mM; dehydro*epi*androsterone, 0·1 mM; and *epi*testosterone, 0·1 mM.

RESULTS

The general validity of the methods of analysis was first demonstrated as follows. A qualitative assay was carried out as described above and after removal of a 0·02 ml. sample of the resulting solution for chromatographic analysis the remainder was diluted to about 6 ml. with water and treated with Zeo-Karb 225 H^+ as described above. The solution was neutralized, evaporated to dryness *in vacuo* and the residue taken up in 0·25 ml. of water. A 0·02 ml. sample of this solution was taken for chromatography and to the remainder was added 0·25 ml. of 2N-HCl. The mixture was heated on a boiling-water bath for 30 min. and then again

taken to dryness. The residue was dissolved in 0·25 ml. of water and a further 0·02 ml. sample was taken for chromatography. Before the resin treatment, spots corresponding to 2-naphthylamine, 2-naphthyl sulphamate and two unidentified metabolites were obtained; after treatment with Zeo-Karb 225 H^+ only the spot corresponding to 2-naphthyl sulphamate remained, whereas after the acid hydrolysis only 2-naphthylamine could be detected. The method is therefore satisfactory in that the resin treatment removed all the metabolites of 2-naphthylamine which could be detected by diazotization, or by Ehrlich's reagent, other than 2-naphthyl sulphamate.

The recovery of 2-naphthyl sulphamate added to the reaction mixture without incubation was approximately constant at 90%. The recovery of the sulphamate after ethanol precipitation of the proteins, as used in the chromatographic experiments, was very poor and could not be increased above about 50%.

General properties of the enzyme system. These studies were carried out with enzyme preparations from rat or from guinea-pig livers, there being no significant differences between the results obtained with the two species. The optimum ATP concentration was 4 mM; the optimum concentration of SO_4^{2-} was 10 mM and the optimum pH was 8·2–8·3 in 0·05M-tris buffers. The optimum substrate concentration could not be determined as it was apparently considerably greater than 2 mM-2-naphthylamine, the highest concentration with which it was possible to work because of the limited solubility of the amine in alkaline solutions.

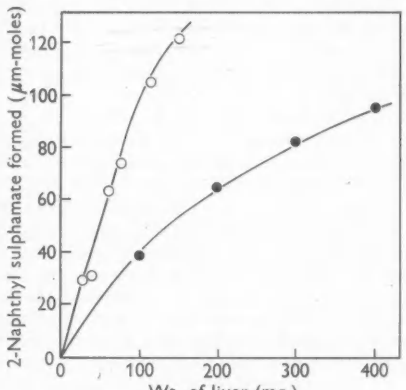

Fig. 1. Effect of varying amounts of enzyme on the rate of synthesis of 2-naphthyl sulphamate. Vol. of reaction mixture 5 ml., containing volumes of enzyme solution corresponding to the specified amounts of liver; pH 8·2 in 0·05M-tris buffers; ATP, 4 mM; SO_4^{2-}, 10 mM; Mg^{2+}, 4 mM; 2-naphthylamine, 2 mM. ○, Guinea pig; ●, rat.

In these conditions there was, with enzyme preparations from guinea-pig liver, a linear relation between the amount of enzyme present and the extent of sulphamate synthesized (Fig. 1) and the reaction was of zero order for at least 2·5 hr. This was not strictly so with rat-liver preparations, with which the reaction velocity did not increase strictly in proportion to the enzyme concentration, as shown in Fig. 1. This behaviour of the rat enzyme was not altered by dialysis of the enzyme preparation against 0·01M-K_2SO_4 in an attempt to remove possible endogenous inhibitors whose presence would account for the shape of the appropriate curve in Fig. 1.

No thorough search for inhibitors has been undertaken, but the synthesis of 2-naphthyl sulphamate is strongly inhibited by EDTA, 0·05M-EDTA inhibiting the reaction by about 99%. This is to be expected, as it is known that the activation of sulphate depends upon the presence of Mg^{2+} ions (Robbins & Lipmann, 1958). As mentioned below, arylamine sulphokinase also requires Mg^{2+} for its full activity.

The substrate specificity of the enzyme system has not been thoroughly investigated, but, as already briefly reported (Roy, 1958), 1-naphthylamine and aniline can also form sulphamates in this sytem although aniline reacted much more slowly. Neither benzylamine nor glucosamine inhibited the synthesis of 2-naphthyl sulphamate; this would suggest that these substances are not substrates for this enzyme system.

Role of adenosine 3′-phosphate 5′-sulphatophosphate. From the generally accepted mechanism of sulphate-ester formation (Lipmann, 1958) it seemed likely that adenosine 3′-phosphate 5′-sulphatophosphate would be an intermediary in the formation of sulphamates. Evidence for this was first obtained when it was shown that neither the sulphate-activating system nor the sulphokinase fraction, prepared as described above, could catalyse the formation of 2-naphthyl sulphamate, but that a mixture of the two fractions did catalyse sulphamate formation. The results of a typical experiment are shown in Table 1, which also shows that the sulphate-activating system from yeast could replace that obtained from liver. This seemed good evidence for the hypothesis that adenosine 3′-phosphate 5′-sulphatophosphate was indeed involved in the synthesis of 2-naphthyl sulphamate.

It was then shown chromatographically that, when a preparation of adenosine 3′-phosphate 5′-sulphatophosphate, obtained as described above, was incubated with the sulphokinase fraction in the

absence of added SO_4^{2-} ions, formation of 2-naphthyl sulphamate occurred. These sulphokinase preparations cannot utilize ATP and SO_4^{2-} ions for sulphamate synthesis (Table 1), so that their presence in the crude preparations of adenosine 3′-phosphate 5′-sulphatophosphate which were available was of no consequence.

From these experiments it therefore seemed justifiable to conclude that sulphate was transferred from adenosine 3′-phosphate 5′-sulphatophosphate to 2-naphthylamine, forming 2-naphthyl sulphamate, by a presumably specific sulphokinase, arylamine sulphokinase. The pH optimum for the reaction was 8·2–8·3 in 0·1 M-tris buffers, identical with that for the overall reaction. The transfer reaction was, as expected, independent of the presence of SO_4^{2-} ions, but its velocity was governed by the concentration of adenosine 3′-phosphate 5′-sulphatophosphate. As shown in Fig. 2, 1 ml. of the standard preparation of adenosine 3′-phosphate 5′-sulphatophosphate described above was sufficient to saturate the enzyme: assuming a quantitative transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate it follows that the concentration of the latter in the solution must be at least 0·25 mM. Arylamine sulphokinase required the presence of Mg^{2+} ions in concentrations of at least 2 mM, before it exhibited its full activity: in the absence of added Mg^{2+} ions the activity was only about 40 % of the maximum.

Arylamine sulphokinase is probably an SH enzyme, as it is inhibited by about 50 % with mM-*p*-chloromercuribenzoate, this inhibition being almost completely reversed by cysteine.

The above results were obtained with both rat and guinea-pig sulphokinase preparations.

Effect of steroids on sulphamate synthesis. If, as indicated above, the synthesis of 2-naphthyl sulphamate involved transfer from adenosine 3′-phosphate 5′-sulphatophosphate then it might be expected that the addition of steroids such as dehydro*epi*androsterone to the reaction mixture would cause a drop in the amount of sulphamate synthesized by competing for the adenosine 3′-phosphate 5′-sulphatophosphate, it being known that the enzyme preparations used in this work can form steroid sulphates (Roy, 1956). With enzyme preparations from guinea-pig liver the addition of dehydro*epi*androsterone to the reaction mixture did in fact cause an inhibition of 2-naphthyl sulphamate synthesis, 0·05 mM dehydro*epi*androsterone causing a 90 % inhibition. It is, however, unlikely that the inhibition is due to a competition for adenosine 3′-phosphate 5′-sulphatophosphate, as dehydro*epi*androsterone sulphate similarly inhibited this reaction and also the synthesis of phenyl sulphamate from aniline.

On the other hand, when the influence of similar steroids on the synthesis of 2-naphthyl sulphamate by rat-liver preparations was studied the very surprising result was an increase in the amount of sulphamate produced. These results are summarized in Table 2 and Fig. 3, the latter showing that the effect was most pronounced at concentrations of approximately 10^{-5} M steroid. At concentrations of less than 10^{-5} M the free steroid and its sulphate ester had approximately similar activating effects, but at higher concentrations the effect of the free steroid fell off more rapidly. The magnitude of the effect varied in different enzyme preparations but its occurrence was quite constant; with a concentration of 10^{-5} M-*epi*androsterone the activation produced varied from approximately 300 to 500 % with different preparations.

In experiments involving the use of free steroids these were added as the thin film obtained by

Table 1. *Participation of the sulphate-activating system and a sulphokinase in the synthesis of 2-naphthyl sulphamate*

The enzyme fractions were prepared and the assays were carried out as described in the text. The activities are expressed as μm-moles of 2-naphthyl sulphamate formed/ml. of enzyme preparation in 2 hr.

Enzyme preparation	Activity
Unfractionated enzyme	140
Activating system	40
Sulphokinase fraction	0
Sulphokinase fraction + activating system	110
Yeast activating system	0
Yeast activating system + sulphokinase fraction	68

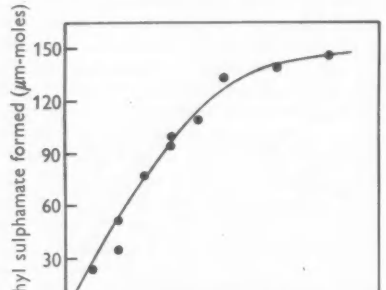

Fig. 2. Effect of varying amounts of the standard adenosine 3′-phosphate 5′-sulphatophosphate preparation (see text) on the rate of synthesis of 2-naphthyl sulphamate by guinea-pig sulphokinase preparations. Vol. of reaction mixture 5 ml., containing enzyme corresponding to 250 mg. of liver; pH 8·2 in 0·1 M-tris buffers; Mg^{2+}, 2·5 mM; 2-naphthylamine, 2 mM.

Table 2. *Influence of steroids and steroid sulphate on the synthesis of 2-naphthyl sulphamate*

The enzyme activity is expressed relative to that in controls with no added steroid (activity 1·0). The steroid concentration was 10^{-5}M and the steroid sulphate concentration 10^{-4}M.

	Steroid	Steroid sulphate
3-Hydroxyoestra-1:3:5-triene	0·9	—
3-Hydroxyoestra-1:3:5-trien-16-one	1·4	—
3-Hydroxyoestra-1:3:5-trien-17-one	1·4	—
3-Methoxyoestra-1:3:5-trien-17-one	1·4	—
3:16α-Dihydroxyoestra-1:3:5-trien-17-one	0·9	—
3β-Hydroxy-5α-androstane	1·1	—
3β-Hydroxyandrost-5-ene	1·0	—
3α-Hydroxy-5α-androstan-17-one (Androsterone)	2·1	2·4
3β-Hydroxy-5α-androstan-17-one (*epi*Androsterone)	3·3	3·4
3α-Hydroxy-5β-androstan-17-one	2·1	2·1
3β-Hydroxy-5β-androstan-17-one	2·2	2·6
3β-Hydroxyandrost-5-en-17-one (Dehydro*epi*androsterone)	2·4	2·9
3β-Acetoxyandrost-5-en-17-one	1·8	—
3β-Methoxyandrost-5-en-17-one	2·5	—
3β:16β-Dihydroxyandrost-5-en-17-one	0·8	—
6β-Hydroxy-3:5-*cyclo*androstan-17-one	1·3	—
17α-Hydroxyandrost-4-en-3-one	0·8	1·1
17β-Hydroxyandrost-4-en-3-one	0·9	0·7
3β:17β-Dihydroxyandrost-4-ene	0·9	—
Androst-4-ene-3:17-dione	1·9	—
11β-Hydroxyandrost-4-ene-3:17-dione	2·5	—
Androst-4-ene-3:11:17-trione	2·1	—
3α-Hydroxy-5α-pregnan-20-one	0·8	0·4
3β-Hydroxy-5α-pregnan-20-one	0·9	0·7
3α-Hydroxy-5β-pregnan-20-one	0·9	0·7
3β-Hydroxy-5β-pregnan-20-one	0·9	0·6
3β-Hydroxypregn-5-en-20-one	0·9	0·5
17α:21-Dihydroxypregn-4-ene-3:11:20-trione	0·9	—
11β:17α:21-Trihydroxypregn-4-ene-3:20-dione	1·0	—

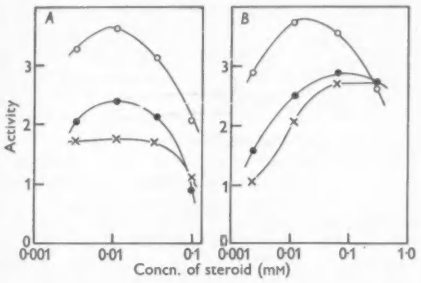

Fig. 3. Influence of steroids (*A*) and steroid sulphates (*B*) on the velocity of 2-naphthyl sulphamate synthesis by rat-liver preparations. Conditions as in Fig. 1, but the enzyme concentration was kept constant at that corresponding to 200 mg. of liver. Activity is expressed relative to controls with no added steroid. ○, *epi*Androsterone and sulphate; ●, dehydro*epi*androsterone and sulphate; ×, androsterone and sulphate.

evaporating to dryness in the reaction tubes a solution of the requisite amount of the steroid in ether. The remainder of the reaction mixture was then added. The low concentrations of steroid required and the relatively prolonged incubation times justified this procedure, which eliminated any possible complicating effects that might have arisen through using a steroid solvent, such as propylene glycol.

From the results summarized in Table 2 it is obvious that of the large number of steroids studied only those having a keto group in the 17 position, or perhaps in the 16 position, exhibited this activating effect on the synthesis of 2-naphthyl sulphamate by rat-liver preparations. Chromatographic experiments showed that the increase was not due to any general increase in the metabolism of 2-naphthylamine, as only the amount of 2-naphthyl sulphamate was increased in the presence of dehydro*epi*androsterone, the amounts of the other metabolites not being altered.

There appeared to be an antagonism between 17-oxo and 17-hydroxy steroids, especially the 17α-hydroxy compound, as these depressed the activating effect of the former. In a typical experiment the presence of *epi*androsterone (10^{-5}M) in the reaction mixture increased the amount of sulphamate synthesized to some 300% of the control value: when testosterone or *epi*testosterone of the same concentration was simultaneously present the activity was respectively only 190 or 250% of the control. The presence of 17-deoxy steroids together with *epi*androsterone had little effect on the activation caused by the latter.

For investigation of the possible mechanism of this activation *epi*androsterone and dehydro*epi*androsterone, or their sulphate esters, were chosen. The free steroids were used at a concentration of 10^{-5} M and the sulphates at 10^{-4} M. The activating effect of all these compounds depended upon the presence of ATP and SO_4^{2-} ions in the reaction mixture; this would appear to eliminate the possibility which was first considered, namely that the sulphate group was being transferred from the steroid sulphate to the 2-naphthylamine. Such a transfer would not be expected to require the presence of SO_4^{2-} ions provided that a sufficient concentration of the steroid sulphate was present. In a system containing a steroid sulphate the ATP could not be replaced by adenosine 3′:5′-diphosphate, which acts as a cofactor in the transfer of sulphate from *p*-nitrophenyl sulphate to phenol (Gregory & Lipmann, 1957). The activation of sulphamate synthesis was apparently a fairly general phenomenon as the rate of synthesis of phenyl sulphamate and of 1-naphthyl sulphamate was also increased by dehydro*epi*androsterone sulphate.

The influence of steroids and of steroid sulphates on the transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to 2-naphthylamine by arylamine sulphokinase was also investigated. The results were similar to those for the overall synthesis of sulphamate from ATP and SO_4^{2-} ions. It therefore follows that the activation of sulphamate synthesis by steroids must be due to an action of the latter on the sulphokinases rather than on the sulphate-activating system. It must again be stressed that the sulphokinase preparations used in these experiments contain sulphokinases other than arylamine sulphokinase and the steroid effect may well be on the former, as is suggested below.

Rates of synthesis of other sulphate esters. The results of these determinations are summarized in Table 3. These figures refer to the overall rate of synthesis of the sulphate ester from ATP and SO_4^{2-} ions. From these data it is obvious that in the rat arylamine sulphokinase must be the limiting enzyme in the synthesis of 2-naphthyl sulphamate, as the enzyme preparations can synthesize *p*-nitrophenyl sulphate at approximately 1 μmole/g./hr., and so presumably can synthesize adenosine 3′-phosphate 5′-sulphatophosphate at a similar rate, yet the rate of sulphamate formation is only about 25% of this. In the guinea pig, on the other hand, arylamine sulphokinase is probably not rate-limiting.

DISCUSSION

The above results show that liver preparations can synthesize 2-naphthyl sulphamate, and related aryl sulphamates, from the parent amine in the presence of ATP and SO_4^{2-} ions. This synthesis probably involves the transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate, a reaction catalysed by arylamine sulphokinase. Although this enzyme has not yet been separated from the other sulphokinases present in the enzyme preparations used there seems no reason to doubt its separate identity; this view is supported by the high specificity of the known sulphokinases (Nose & Lipmann, 1958) and by the comparative data summarized in Table 3. Arylamine sulphokinase is further distinguished from phenol sulphokinase by the requirement of the former for Mg^{2+} ions, the activity of phenol sulphokinase apparently being independent of the presence of these ions (Gregory & Lipmann, 1957), although the results of Segal (1957) do not confirm this latter claim.

The most interesting observation is the pronounced activation of the synthesis of 2-naphthyl sulphamate by 17-oxo steroids. As yet it has not proved possible to elucidate completely the mechanism of this activation, but a reasonable hypothesis would seem to be that the steroids are functioning in their corresponding enolic forms. Some evidence for this is given in Table 2, which shows that 16-hydroxy 17-oxo steroids, which cannot enolize, do not activate the synthesis of sulphamate. A possible explanation for this activation would seem to be provided by the following reaction sequence:

$$SO_4^{2-} \longrightarrow \text{adenosine 3′-phosphate 5′-sulphatophosphate} \begin{cases} \nearrow \text{17-enol sulphate} \\ \searrow \text{2-naphthyl sulphamate} \end{cases}$$

The compound primarily responsible for the increase in the amount of sulphamate formed is presumed to be the 17-enol sulphate, formed enzymically from adenosine 3′-phosphate 5′-sulphatophosphate by a reaction catalysed either by a general steroid sulphokinase or, more probably, by a specific 17-hydroxy steroid sulphokinase. Rat-liver preparations, similar to those used in the present experiments, can form steroid 17-sulphates from 17-hydroxy steroids (Roy, 1956; Schneider & Lewbart, 1956) and it may be that the sulphokinase involved could also yield 17-enol sulphates under suitable conditions. Such a 17-enol sulphate would be expected to have a very high sulphate-group potential, comparable with the phosphate-group potential of phosphoenol pyruvate, and it might well be able to transfer its sulphate group to an acceptor such as 2-naphthylamine to form the sulphamate in which the sulphate-group potential

Table 3. *Rates of synthesis of sulphate esters by liver preparations*

The general conditions of assay were as described in the text and the rates of synthesis are expressed as μm-moles synthesized/g. of liver/hr.

	Rat		Guinea pig (male and female)
	Male	Female	
p-Nitrophenyl sulphate	1500	1200	370
Oestrone sulphate	78	47	72
Dehydro*epi*androsterone sulphate	120	390	260
*epi*Testosterone sulphate	59	200	150
2-Naphthyl sulphamate	140	320	1200

would certainly be relatively low. No mechanism can yet be suggested for such a transfer; but it cannot involve arylamine sulphokinase, as it is obvious from the data of Table 3 that this enzyme is the rate-limiting one in the synthesis of sulphamate by rat-liver preparations.

* The test of this theory would be the synthesis of a 17-enol sulphate and its use as a sulphate donor in the absence of adenosine 3′-phosphate 5′-sulphatophosphate. Unfortunately this has not so far proved possible, as attempts to prepare these compounds chemically have failed and there is no reason to assume that they would accumulate in the enzyme systems used above.

The above sequence of reactions could also account for the failure of 17-oxo steroids to activate the synthesis of 2-naphthyl sulphamate by guinea-pig-liver preparations. As pointed out above, in such preparations the synthesis of adenosine 3′-phosphate 5′-sulphatophosphate is probably the rate-limiting reaction in the overall synthesis of 2-naphthyl sulphamate; if this indeed be the case then activation by the mechanism postulated above could not occur. On the other hand, the synthesis of phenyl sulphamate by guinea-pig preparations is a much slower reaction than the synthesis of 2-naphthyl sulphamate, so that in the former case arylamine sulphokinase must be rate-limiting; activation of phenyl sulphamate synthesis by steroids might therefore be expected. As reported above, dehydro*epi*androsterone sulphate inhibits the synthesis of phenyl sulphamate by guinea-pig enzymes; this would suggest that some other explanation must be sought for the failure of steroids to activate sulphamate synthesis in preparations from this species. The reason might lie in the absence of a sulphokinase capable of forming steroid 17-enol sulphates in guinea-pig liver, but no definite evidence on this point is available at present.

It cannot yet be stated whether or not this steroid activation of sulphamate synthesis in the rat has any physiological significance. The concentrations of steroids used are close to the physiological; but the significance of aryl sulphamate synthesis in the normal animal is obscure, as no such compounds have ever been detected therein. Should steroids have a similar action on the synthesis of the sulphamate group of heparin, however, then the mechanism may well be of considerable physiological importance, as steroids are known to influence mucopolysaccharide metabolism *in vivo*. Aryl sulphamates have been used as sulphating agents *in vitro* (Brodersen & Quaedvlieg, 1933); it is therefore possible that aryl sulphamates might have a similar action *in vivo*, forming sulphate esters by a mechanism not directly involving adenosine 3′-phosphate 5′-sulphatophosphate. There is, of course, no evidence for this at present.

Perhaps the most important point arising from this work is the suggestion that forms of 'active sulphate' other than adenosine 3′-phosphate 5′-sulphatophosphate may exist and that a steroid 17-enol sulphate may be an example of such a compound with a high sulphate-group potential.

SUMMARY

1. A method is described for the assay of 2-naphthyl sulphamate synthesis by liver preparations. The determination of the sulphamate is based on the removal of free amino compounds by treatment with ion-exchange resin, hydrolysis of the sulphamate to the amino compound and its subsequent determination by diazotization and coupling with thymol.

2. The optimum pH of the reaction is 8·2, the optimum concentration of adenosine triphosphate is 4 mM, the optimum sulphate concentration is 10 mM, and the optimum concentration of 2-naphthylamine is greater than 2 mM.

3. 1-Naphthylamine and aniline are also substrates for this enzyme system, but benzylamine and glucosamine are not.

4. The sulphamate is probably formed by the transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate, the transfer being catalysed by arylamine sulphokinase, which has an optimum pH of 8·2 and requires the presence of at least 2 mM-Mg^{2+} ions before it shows its activity. The enzyme is inhibited by *p*-chloromercuribenzoate and the inhibition abolished by cysteine.

5. The rate of synthesis of 2-naphthyl sulphamate by rat-liver preparations is increased by about 400 % by 17-oxo steroids in concentrations of approximately 10^{-5} M.

6. The rate of synthesis of sulphamate by guinea-pig preparations is not increased by 17-oxo steroids.

7. It is suggested that the mechanism of this activation involves the formation of steroid 17-enol sulphates and that these enol sulphates would be a form of 'active sulphate', having a high sulphate-group potential.

8. The rates of synthesis of certain other sulphate esters by the enzyme preparations are described.

The author wishes to express his gratitude to Miss Pamela Burton for carrying out many of the preliminary experiments and for making the observations which led to the discovery of the steroid effects. He is also indebted to Miss Isla Sharp for her skilled technical assistance throughout the work.

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The Cholesterol Esters Circulating in Human Blood in Health

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The free and esterified cholesterol circulating in human blood has frequently been measured but, because of the experimental difficulties involved, scant attention has been paid to the fatty acids combined with cholesterol. Kelsey & Longnecker (1941), using selective enzymic hydrolysis of the esters (Kelsey, 1939*a*, *b*, *c*), identified some of the esters in the serum of lactating cows and, with chromatographic methods, Lough & Garton (1957) have been able to demonstrate the presence of many more. It seemed possible that knowledge of the identity of the fatty acid component of these esters would throw some light on the metabolism of cholesterol in the human.

In the present work, chromatographic methods were employed to isolate the cholesterol esters from the lipids of normal human sera, to establish their identity and estimate the amount of each fatty acid. The results obtained indicate that over 70 % of the acids are unsaturated and more than half of these are polyethenoid.

* Some of this work formed part of a thesis submitted to the University of Edinburgh for the degree of M.D.

EXPERIMENTAL

Materials

Most of the fatty acids used in examining the characteristics of the columns were prepared from commercial specimens by fractional distillation of their methyl esters at pressures of about 0·1 mm. Hg. The commercial samples of stearic acid were, however, found to be heavily contaminated with palmitic acid. A sample of high purity was eventually prepared by hydrogenation of commercial elaidic acid (L. Light and Co. Ltd.). A sample of pure linoleic acid was kindly provided by Unilever Research Laboratories, Port Sunlight, Cheshire. All solvents were of reagent grade except for the ether, which was of micro-analytical reagent grade. The acetone was slightly acid and the resulting small titration blank was allowed for in the determination of the fatty acids.

Blood samples. Two samples of serum and two of plasma were analysed. Sample I consisted of 100 ml. of pooled sera from 25 patients, none of whom was suffering from liver disease. Sample II was 25 ml. of serum from a healthy male aged 39 years. Sample III was 400 ml. of plasma from two separate pints of transfusion-bank blood; this sample was stored at 0° for 6 weeks before it was extracted. Sample IV was 200 ml. of plasma obtained from another

pint of transfusion-bank blood; it was stored at 0° for 2 days before it was extracted. Nothing was known of the donors of the plasma but it may be safely assumed that they were healthy. Samples III and IV had both been collected into disodium citrate–dextrose solution.

Methods

Lipid-extraction procedure. Each sample of serum was poured into ten times its volume of ethanol and allowed to stand overnight at room temperature. It was then filtered and the protein residues were dispersed in chloroform–methanol (1:1, v/v) equal in amount to the original volume of serum, refluxed for 3 hr. and then filtered. The extraction with chloroform–methanol was repeated and after the final filtration the residues were washed twice with chloroform. All extracts were pooled and evaporated to small volume under reduced pressure, the temperature being kept below 60°. The concentrated liquor was extracted three times with light petroleum (40–60°) and the combined extracts were dried over anhydrous sodium sulphate.

Sample III was poured into its own volume of ethanol and allowed to stand overnight. After filtration, the protein residues were refluxed with ethanol–ether (1:1, v/v) equal in amount to the original volume of plasma. After filtering the mixture again, the protein residues were refluxed with chloroform–ethanol–methylal (1:1:1, by vol.) for 4 hr. A subsequent extract contained no cholesterol and was discarded.

Sample IV was refluxed for 2 hr. with 6 vol. of acetone–methanol (5:7, v/v) and, after filtering, this process was repeated on the protein residues. The residues were once more extracted with methanol–methylal–chloroform (2:1:1, by vol.) equal to twice the original volume of plasma and finally with twice its volume of ethanol–chloroform–light petroleum (1:1:1, by vol.). The cholesterol recovered in the last fraction amounted to 0·3 mg. (by the Lieberman–Burchard estimation). All fractions were combined and evaporated to dryness under reduced pressure.

To ensure that no cholesterol remained entrapped in the protein residues from samples I, II and III they were separately incubated overnight with 25% (w/v) potassium hydroxide to hydrolyse the protein. The excess of alkali was neutralized with dilute hydrochloric acid and the mixture was extracted repeatedly with light petroleum (40–60°). Quantitative Lieberman–Burchard estimations on the residues from the combined light petroleum extracts showed only traces of cholesterol, ranging from 0·05 to 0·60 mg.

Chromatographic separation of the cholesterol esters. Columns of silicic acid were used (Fillerup & Mead, 1953, 1954). Silica gel (chromatographic grade; 200–300 mesh) gave good separation of an artificial mixture of lipids consisting of cholesterol, cholesterol palmitate, cholesterol oleate and cholesterol stearate, triolein, tristearin and tripalmitin, and egg lecithin (British Drug Houses Ltd.). Satisfactory flow rates were obtained without the admixture of filter aids. In the actual separation of blood lipids, 30 g. of silicic acid was used, giving columns of about 1·8 cm. bore by 30 cm. long. Previous experiments with the artificial mixtures showed that these columns separated satisfactorily lipid mixtures of up to 900 mg., i.e. 30 mg./g. of silica gel. The silicic acid was transferred to the column as a slurry in light petroleum (40–60°) and packed by mechanical vibration. Lipid mixtures from the plasma and serum extracts were transferred to the columns in the minimum volume of light petroleum (40–60°) and washed in with a further small volume of the same solvent. This was followed by 300 ml. of the same solvent, after which 1% (v/v) diethyl ether–light petroleum (40–60°) was used to elute the cholesterol esters. Fractions of 50 ml. were collected and 1 ml. samples were examined for cholesterol by the Lieberman–Burchard test, elution being continued until the result of this test became negative. In sample I the triglycerides, free cholesterol and phospholipids were separated and three 1 ml. samples were removed from each 50 ml. fraction and examined for cholesterol, glycerol and phosphorus. In the other three samples, phosphorus and glycerol analyses were carried out only on a portion of the combined Lieberman–Burchard-positive 1% ether–light petroleum fractions.

Reversed-phase chromatography of fatty acids. Columns were first prepared in the manner described by Howard & Martin (1950) but proved unsatisfactory; the stationary phase was flocculent and difficult to pack and the columns had exceedingly slow flow rates; furthermore, when fatty acids in acetone solution were added to the columns the stationary phase collapsed and shed liquid paraffin. This behaviour appeared to be due to the small particle size of the kieselguhr that was used at this stage. Columns were therefore prepared as described by Silk & Hahn (1954), with preliminary removal of the fine particles by suspension in water. These columns gave satisfactory separation of artificial mixtures of fatty acids. Unsaturated acids were added to the columns in solution in acetone–water, but saturated acids were generally too insoluble and were made up into a small quantity of the mull as recommended by Silk & Hahn (1954). Acetone–water mixtures equilibrated with liquid paraffin were used to elute the fatty acids: the mixtures were stored at 37° when not in use. For sample I the concentration of the acetone was increased by steps of 2·5% between 45 and 80% (v/v), but 5% increments were used in the subsequent work without affecting the sharpness of the separations. Fractions (5 ml.) of the eluate were collected and titrated with 0·02N-tetramethylammonium hydroxide in aqueous 50% (v/v) ethanol in a Conway burette, 0·1% bromothymol blue being used as indicator; the alkali was standardized daily against 5 mN-benzoic acid in ethanol. Columns were of 1·8 cm. bore and were packed with 25–35 g. of stationary phase. Firm packing was found to be important and was effected by lowering a pad of cotton wool on to the surface of the kieselguhr and ramming with a stainless-steel plunger. The columns were enclosed in a tinplate jacket of 3 in. diameter which was heated by a 50 w heating element (Isotape) wrapped round the outer surface. The temperature was controlled by an oven-type bimetallic thermostat to between 33° and 36°.

Hydrolysis. The combined cholesterol ester fractions were washed with water and evaporated to dryness; the residue was dissolved in 10 ml. of diethyl ether and 10 ml. of 3M-sodium ethoxide was added. Water (10 ml.) was then added to reduce the risk of isomerization and the mixture was refluxed in an atmosphere of nitrogen. After acidification of the reaction mixture with dilute hydrochloric acid the fatty acids were extracted with light petroleum, the combined extracts were washed with water, dried over sodium sulphate and evaporated to dryness in a stream of nitrogen.

Hydrogenation. Samples of fatty acids (10–20 mg.) from the hydrolysis mixture in ethyl acetate solution were hydrogenated at 1·1 atmosphere pressure with 10% palladized charcoal. Constant agitation for 48 hr. with 50 mg. of catalyst was necessary to ensure complete hydrogenation.

Oxidation. Another 10–20 mg. sample of the fatty acids was treated with alkaline potassium permanganate at room temperature for 18 hr. (Crombie, Comber & Boatman, 1955). At the end of this time the iodine value of the product was still about 20, probably because partial conjugation induced during hydrolysis prevented full hydroxylation of the polyethenoid acids. No further reduction in iodine value was obtained if oxidation was continued for 48 hr. Nevertheless, as a precautionary measure to ensure the fullest possible oxidation, all the samples were oxidized for at least 48 hr. The product was then examined by reversed-phase chromatography. In no case was any acid eluted in 50–60% acetone, indicating that all polyethenoid acids had undergone some degree of oxidation.

Ester and free cholesterol. Ester and free cholesterol were estimated by the method of Obermer & Milton (Milton & Waters, 1955).

Glycerol. Glycerol was estimated by the method of Lambert & Neish (1950) after hydrolysis with 25% potassium hydroxide in 50% ethanol at 100° for 1 hr. The accuracy of the method was tested with tristearin and complete recovery of glycerol was obtained.

Iodine value. Iodine values were determined by the method of Yasuda (1931).

Phosphorus. The method of Fiske & Subbarow (1925) was used for the estimation of phosphorus, after wet ashing with sulphuric acid.

Alkaline isomerization. Alkaline isomerization was carried out by the method of Holman (1957) and the extinctions were measured in a Unicam SP. 500 spectrophotometer.

RESULTS

Separation of blood total lipids. Fig. 1 shows the results of the cholesterol, phosphorus and glycerol analyses on fractions recovered from the silicic acid column for sample I. A small amount of phosphorus-containing material was eluted early, in pure light petroleum. This material gave ammonia on incubation with urease, and glycerol was obtained after prolonged alkaline hydrolysis. This fraction was observed for all samples except the last one; in this, the solvent mixtures used for extraction of the plasma lipids contained a high proportion of acetone. Presumably this lipid is either a glycerophosphatide–urea complex or else urea dissolved in a solution of glycerophosphatides.

Separation of cholesterol ester fatty acids. The cholesterol esters recovered from the silicic acid columns were weighed and hydrolysed and the recovered fatty acids titrated. Table 1 shows the comparison of the yields obtained with the expected recoveries calculated from analysis by digitonin precipitation, carried out on the original plasma samples. A sample of the fatty acids was separated by reversed-phase chromatography. The

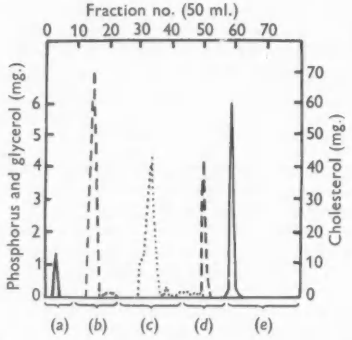

Fig. 1. Elution pattern of mixed serum lipids on a column of silicic acid. (*a*) Light petroleum; (*b*) 1% of ether in light petroleum; (*c*) 3% of ether in light petroleum; (*d*) 10% of ether in light petroleum; (*e*) ether–methanol (1:1, v/v). —, Phosphorus; - - -, cholesterol; ....., glycerides.

peaks obtained were similar in all experiments and a representative picture of their chromatographic pattern is shown in Fig. 2. There are two large clearly defined peaks, *A* and *B*, eluted by 60 and 65% acetone respectively, and a third smaller peak, *C*.

The small peak eluted in 45–50% acetone was not observed in the later experiments. It was present only when the light-petroleum extract was not washed thoroughly with water and it seems probable that it represents a trace of the hydrochloric acid used to convert soaps into free acids.

The acids comprising peak *A* were recovered and hydrogenated and the resulting saturated acids were again examined by reversed-phase chromatography (Fig. 3). There is a small rather irregular peak *D* eluted with 65% acetone corresponding to palmitic acid, a larger well-defined peak *E*, eluted with 75% acetone, and a small ill-defined peak *F* eluted with 80% acetone and corresponding to arachidic acid.

The irregular shape of peak *D* was also observed when the columns were tested with samples of pure acids. Palmitic acid is eluted slowly from these columns with 65% acetone, forming a low plateau rather than a peak. It appears probable that, under these conditions, the 3° fluctuation of temperature permitted by the thermostat in use is sufficient to cause deformation of the elution curve. The acids of peak *B*, after similar treatment, gave the results shown in Fig. 4. There are clearly defined peaks *G* and *H*, corresponding to palmitic acid and stearic acid respectively.

Another portion of the mixed acids obtained by hydrolysis was treated with alkaline potassium

permanganate. The result of reversed-phase chromatography of the oxidation products is shown in Fig. 5. The fraction eluted with 45% acetone contains all the oxidation products, and the small peaks *N*, *O* and *P* represent the unchanged palmitic acid, stearic acid and arachidic acid present in the original mixture.

Figs. 3 and 4 show that peak *A* consists of acids containing 16, 18 and 20 carbon atoms and that peak *B* consists of acids containing 16 and 18 carbon atoms. The C_{18} acid in peak *A* is eluted in 60% acetone, which is the expected behaviour of linoleic acid. Similarly, the C_{18} acid in peak *B* is eluted in 65% acetone, which is characteristic of oleic acid. The C_{20} acid present in peak *A* is even more unsaturated than the linoleic and must therefore be trienoic or tetraenoic. This acid was presumed to be arachidonic since this is the most widely distributed natural C_{20} polyene and, moreover, alkaline isomerization gave evidence of its presence.

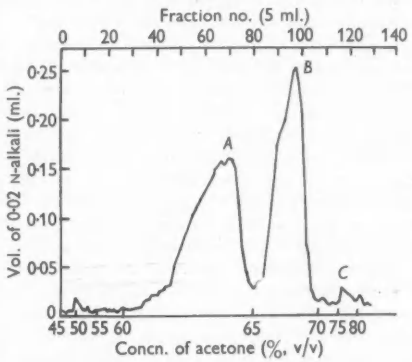

Fig. 2. Reversed-phase chromatography of fatty acids (total fatty acids 59 mg.).

When molar percentages of the various acids comprising each peak were calculated from these results (Table 2) it was found that the palmitic acid remaining after oxidation was approximately equal to the sum of the C_{16} acids present in peaks *A* and *B*. In sample III there was insufficient material on which to carry out the oxidation experiment, so for the purposes of calculation it was assumed that, as was the case in the two previous samples, all the C_{16} acid present was saturated. Since iodine values calculated on this assumption agreed closely with the observed values it seems

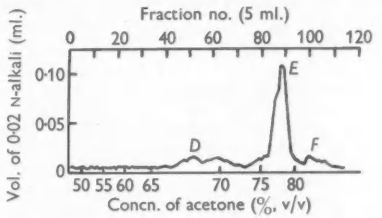

Fig. 3. Reversed-phase chromatography of fatty acids from peak *A* of Fig. 2 after hydrogenation (hydrogenated acids 5·7 mg.).

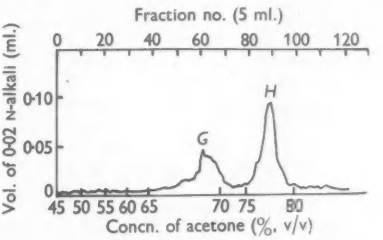

Fig. 4. Reversed-phase chromatography of fatty acids from peak *B* of Fig. 2 after hydrogenation (hydrogenated acids 9·6 mg.).

Table 1. *Comparison of yields of cholesterol ester fatty acids from silica-gel chromatography with expected yields calculated from analysis by digitonin precipitation*

		Sample no. I	II	III	IV
Cholesterol levels (mg./100 ml. of serum) calc. as free cholesterol from analysis by digitonin precipitation	Total	147	105	156	312
	Free	56·2	35	62	96
	Ester	90·8	70	94	216
Total cholesterol ester (mg.) calc. as cholesterol oleate, from analysis by digitonin precipitation		153	529	39·6	366
Wt. of ester (mg.) recovered from silicic acid columns		230	544	46	396
Theoretical wt. of fatty acid (mg.) calculated as oleic acid from analysis by digitonin precipitation		66	229·5	17·1	159
Acid (as oleic) recovered from hydrolysis of cholesterol esters from silicic acid columns	By titration	65·9	240	18	140
	By wt.	—	236	—	—

probable that this assumption is essentially correct. In sample IV peak *A* was larger and contained a larger amount of C_{16} acid. After hydrogenation the amount of saturated C_{16} acid was much greater than in the other three samples. On the other hand, after oxidation, the amount of saturated C_{16} acid remaining was substantially less than the total found after hydrogenation; therefore one must conclude that this sample contained an unsaturated C_{16} acid. Moreover, the close agreement between the observed iodine values and the theoretical values calculated on the basis that this acid is monoethenoid indicates that this sample, unlike the previous ones, contained palmitoleic acid.

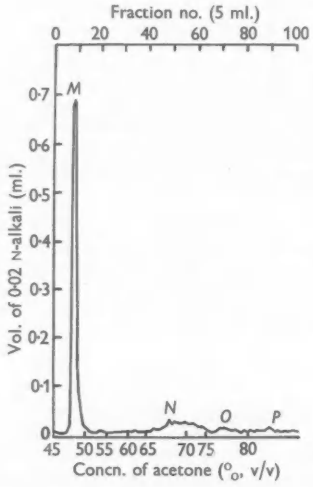

Fig. 5. Reversed-phase chromatography of total acids after oxidation (24 mg.).

Table 2. *Relative proportions of cholesterol ester fatty acids deduced from reversed-phase chromatography (molar percentages)*

Acid	Sample no. I	II	III	IV
Palmitoleic	—	—	—	19·1
Oleic	33·7	29·6	36	28·7
Linoleic	25·3	39	31·6	41·7
Arachidonic	7·1	3·5	7·3	6·1
Other C_{20} unsaturated acids	9·3	—	—	—
Palmitic	18·4	23·5	17	2·2
Stearic	7·8	3	4	2·3
Arachidic	1·6	1	—	—
Total	103·2	99·6	95·9	100·1

Iodine values were determined on the total acids recovered from hydrolysis and on the acids recovered from peaks *A* and *B* (Fig. 2). Comparison between the observed values and theoretical values calculated from the chromatographic data showed close agreement within the limits imposed by the small amounts of material available. In addition, alkaline isomerization was carried out on the acids recovered from samples III and IV and figures for total polyethenoid acids agreed closely with those obtained by chromatography. Alkaline isomerization also showed evidence of small amounts of linolenic acid, eicosapentaenoic acid and docosahexaenoic acid.

DISCUSSION

The high degree of unsaturation of the blood-cholesterol fatty acids was the most noteworthy finding in this work. Previous workers in this field (Bloor, Blake & Bullen, 1937; Clément, Clément & Louedec, 1954) have drawn attention to the relationship of the plasma-cholesterol esters to the phospholipids and triglycerides, the former being the most unsaturated and the latter the least.

The character of the fatty acids identified in this work appears to throw some light on the question of the site of esterification. It has long been known that some degree of esterification occurs in the intestine (Mueller, 1915, 1916) and the esterases of pancreatic juice have recently been studied in detail (Swell & Treadwell, 1955); work by Glover, Green & Stainer (1959) suggests that esterification occurs in the connective tissues of the gut wall. The cholesterol which reaches the gut is derived either from the biliary secretion or from the diet, the former being entirely free and the latter, being almost completely of intracellular origin, is also almost entirely free. Some 60–80% of the cholesterol reaching the blood via the thoracic duct is esterified (Bollman & Flock, 1951). The predominant unsaturated fatty acid in the human diet is oleic acid and saturated acids form at least 50% of the total. On the other hand, linoleic acid forms only a small proportion of the dietary fat. In the plasma samples examined in this work the linoleic acid of the cholesterol esters is approximately equal in amount to the oleic acid whereas the saturated acids formed only 4·5–27·8% of the total. It appears therefore that the greater the number of double bonds that a dietary fatty acid contains, the greater the likelihood of it being esterified with cholesterol. This view is supported by the fact that the rate of esterification by the intestinal cholesterol esterases is greater the longer the chain length of the acid esterified, and is still greater when unsaturation is present; oleic acid, for example, is esterified at almost twice the rate of stearic acid (Swell & Treadwell, 1955).

In this work no fatty acid of chain length shorter than C_{16} was found, although lauric acid and myristic acid are present in significant amounts in the diet and, according to Swell & Treadwell (1955), are esterified with cholesterol at almost the same rates as stearic acid and palmitic acid. This raises doubt whether the saturated acids are esterified with cholesterol in the gut or in other sites such as the liver and blood. Cholesterol esterase activity does not appear to have been demonstrated in human liver although such activity has been shown in animals (Byron, Wood & Treadwell, 1953). On the other hand, cholesterol esterase activity has been demonstrated in human blood (Sperry, 1935, 1936; Tayeau & Nivet, 1956). It seems possible therefore that whereas the unsaturated acids are probably esterified with cholesterol in the gut, esterification of the saturated acids may take place in the blood. If saturated and unsaturated acids are esterified at different sites it may well be that the saturated and unsaturated esters of cholesterol have different functions.

SUMMARY

1. The cholesterol esters of two samples of human serum and two samples of human plasma have been separated from other lipids, by chromatography on silicic acid, and their component acids have been characterized by reversed-phase partition chromatography.

2. Unsaturated acids formed a high proportion of the total acids present.

3. The relationship between the pattern of the dietary fatty acids and the plasma-cholesterol ester fatty acids is discussed.

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A Study of the Protein Impurities in Gelatins with Ion-Exchange Resins

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From his work on mammalian collagens and gelatins, Eastoe (1955) concluded that the amino acid composition of a gelatin may be regarded as representative of a highly purified form of the collagen from which it was prepared. The amino acid composition of the gelatins derived from various mammalian sources is remarkably constant, which suggests that the proportion of impurity proteins is small. Purification of the gelatin by ethanolic coacervation (Stainsby, 1955), followed by deionization with mixed-bed resins (Janus, Kenchington & Ward, 1951), was shown to produce only very small changes in composition (see Table 6). Certain of the amino acids present in large

amounts in the original gelatin increased slightly in percentage whereas those present in small amounts decreased. The variation of the most abundant amino acids, e.g. glycine, proline and hydroxyproline, suggests that at most only a few per cent of other proteins were present in the original gelatin.

The high purity of gelatin as a protein was disputed by Russell (1958), who reported the presence of two chemically contrasted protein components in lime-processed gelatins. He stated that about 15% of a second component could be isolated from lime-processed gelatins by use of the ion-exchange resin Amberlite IRC-50 (XE-97) and the chromatographic technique of Boardman & Partridge (1955). He also stated that the amino acid composition of this second component differed markedly from that of gelatin, being low in proline, hydroxyproline, glycine and hydroxylysine, and high in tyrosine; it broadly resembled that of the albumins.

This finding, if confirmed, would be of considerable interest owing to its bearing on the interpretation of much previous work, physical and chemical, on gelatins and collagens. It was therefore decided to attempt to repeat the work, to examine the influence of various factors on the eluted peak, and to examine quantitatively the amino acid composition of the eluted peaks.

MATERIALS

Gelatin no. 127. Lime-processed ox-hide gelatin, the same sample as ox-hide II, unpurified, in Eastoe (1955), whose amino acid analysis is given in Table 6. Amide content: 0·16 g. of N/100 g. of dry ash-free protein; p*I* 4·95.

Gelatin no. 142. Acid-processed bone (ossein) gelatin. Amide content: 0·50 g. of N/100 g. of dry ash-free protein; p*I* 7·8; produced commercially from demineralized bone by giving it a brief soak in lime water, followed by washing with water and soaking in dilute acid. The material was washed with water until it reached pH 5·0, when it was extracted with warm water. The short lime treatment produced a gelatin of p*I* value between the extremes for lime-processed and acid-processed gelatins.

Gelatin no. 149. Acid-processed pig-skin gelatin. Amide content: 0·62 g. of N/100 g. of dry ash-free protein; p*I* 9·4. Similar to the acid pig-skin in Eastoe (1955). The amide and p*I* values were determined by J. E. Eastoe, J. E. Long and A. L. D. Willan (personal communication).

Fractionated gelatin no. 127. Two fractions from a triple-fractionation procedure [kindly supplied by A. M. Kragh and P. R. Saunders, who also determined the limiting viscosity numbers (L.V.N.)], using alcoholic coacervation, were employed in the present work. These were gelatin 127/2/3/2, L.V.N. 86·5, and gelatin 127/4/3/4, L.V.N. 55·5: the parent gelatin had L.V.N. 62. From the relationship between M_w (weight av. mol.wt.) determined by light-scattering and L.V.N. for fractions of gelatin no. 127 determined by Courts & Stainsby (1958), the gelatins have M_w 320 000, 122 000 for the fractions and 220 000 for the original gelatin respectively. By comparison with related fractions Kragh & Saunders showed that 127/4/3/4 is homogeneous and 127/2/3/2 rather less so, as far as behaviour in alcoholic fractionation and dilute solution viscosity (i.e. molecular weight) are concerned. The methods used for the determination of M_w by light-scattering and of L.V.N. values are described by Courts & Stainsby (1958).

Resins. The Amberlite resins IRC-50 (XE-97), nominal 200–400 mesh, and IRC-50 (XE-64), nominal 100–200 mesh, were treated in the manner described by Boardman & Partridge (1955), except that the former resin was allowed to settle for 1 hr. These resins will be referred to as XE-97 and XE-64 throughout this paper; XE-97 was the resin used unless specified otherwise.

Buffers. McIlvaine buffers (citric acid–Na_2HPO_4) were made up as described in Vogel (1957), final adjustments being made with 0·2 M-Na_2HPO_4 or 0·1 M-citric acid, usually the latter. For buffers in the region of pH 5·5 the buffer pH must be accurate to within $\pm$0·01.

METHODS

Experiments with small columns

Preparation of columns. Before a chromatography column was poured, the resin was washed twice with about 20 times its own volume of the buffer to be used for elution. A column 16–17 cm. high and 1 cm. in diameter was poured and the resin equilibrated with a large volume of the McIlvaine buffer. During the equilibration the column shrank to about 14–15 cm. high. This column will be referred to as the small column throughout this paper. The larger columns were prepared in a similar manner.

Elution and fractions collected. The selected quantity of the gelatin, usually as a 0·5% solution in the same buffer, was added to the column, allowed to sink into the resin and rinsed in with more of the buffer. The column was then developed with the same buffer at a flow rate of about 4 ml./hr. Fractions of about 1·8 ml. were collected; 1 ml. was used for the protein analysis, leaving 0·8 ml. for dilution if necessary.

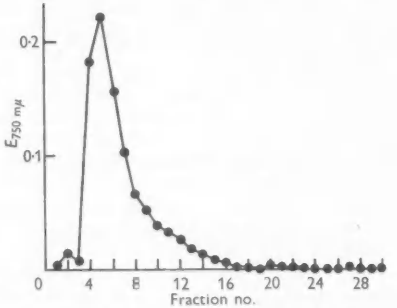

Fig. 1. Elution of 10 mg. of lime-processed gelatin no. 127 on a 14·5 cm. × 1 cm. diameter column of Amberlite IRC-50 (XE-97) resin, which was eluted at 40° with McIlvaine buffer, pH 5·55. The fraction size was 1·8 ml., 1 ml. of which was used in the Lowry, Rosebrough, Farr & Randall (1951) determination of protein. A 2 cm. cell was used.

Analysis of fractions. The fractions were analysed for protein by the Lowry, Rosebrough, Farr & Randall (1951) method. Gelatin was used as the reference protein for the preparation of a standard curve, which would lead to an overestimation of albumin-type proteins by a factor of about 2:1 (Lowry *et al.* 1951). The standards were prepared in the same buffer as that used with the column. A small volume of N-NaOH solution was added to 1 ml. of the fraction, before analysis, to bring the pH to about 7·0.

Typical experiment. A typical experiment was carried out at 40° with 10 mg. of lime-processed gelatin (no. 127), at pH 5·55. on a small column. It yielded 6·6% of eluted material, which appeared as a small peak (Fig. 1) at fraction 4, corresponding to a volume of 5·4 ml., i.e. close to the hold-up volume of the column. The peak was completely eluted at fraction 19, and continued elution up to 200 fractions did not elute any more material. On raising the pH with 0·05N-NaOH, a large peak containing the bulk of the remaining material was eluted. This behaviour closely resembles that reported by Russell (1958). The first peak will be referred to as the eluted peak and is given as a percentage of the original material placed on the column.

Variation of procedure

Temperature. Elutions were carried out with 10 mg. of gelatin no. 127 on short columns at pH 5·52 at each of the temperatures 25°, 32·5° and 40°.

Column loading. Column loadings of 10, 50 and 100 mg. of gelatin no. 127 at pH 5·25, 5–135 mg. at pH 5·5 and 0·125–12·5 mg. at pH 5·75 were used on small columns. Some experiments employed large columns and the equivalent loading on a small column was then calculated.

Resin batch. In addition to the experiments described above with the XE-97 resin, two were carried out with small columns of XE-64 resin, which is known to differ from the former resin only in surface area per unit volume. Loadings of 2·5 and 5·0 mg. of gelatin no. 127 were used at pH 5·5.

pH. A constant loading of 10 mg. of gelatin no. 127 per small column was used with buffers pH 3·0–6·0.

Type of gelatin. Three gelatins (nos. 127, 142, 149; 10 mg. loads) were used on small columns, at pH values 5·5, 5·6 and 6·0 for each gelatin.

Molecular weight of gelatins. Loadings of 10 mg. of the fractions described earlier, on small columns at pH 5·5 and 5·6, were used.

Large-scale separations

Elutions of peaks were carried out on a preparative scale, with similar procedures as for the small columns, to give sufficient material for analysis. The slight differences in procedure are most conveniently given with the results from these experiments.

Hydroxyproline determinations. After acid hydrolysis for 24 hr. at 100° with 20% (w/w) HCl, the materials from the large-scale separations were examined for hydroxyproline content by the following methods:

(1) By noting the presence of hydroxyproline during the 100 cm. chromatographic analysis (Moore & Stein, 1951) for other amino acids. When using the citrate buffer, pH 3·42, with the normal procedure the hydroxyproline appears on the leading edge of the aspartic acid peak, and its presence is masked and can be detected only when appreciable amounts are present. Separate determinations of hydroxyproline were carried out with citrate buffer, pH 3·19, and the 100 cm. column, under which conditions hydroxyproline travels between and separate from methionine sulphoxide and aspartic acid. Hydroxyproline could not be detected in fractions of the 0·36% material by these methods. The reduced column loadings which had to be used, the low colour yield of the hydroxyproline reaction with ninhydrin, and small variations of the buffer blanks from the column in this region made the methods rather insensitive to traces of hydroxyproline.

(2) By use of the Neuman & Logan (1950) technique, as modified by Leach (1959). The values quoted in Table 5 (columns *A*, *B*, *C*) and Table 6 (columns *C*, *D*, *E*) were determined by this method.

Amino acids. The hydrolysates were examined for amino acids by the Moore & Stein (1951) technique as described by Eastoe (1955).

Hexosamines. The hexosamines were determined on the 15 cm. Dowex columns during the analyses for basic amino acids (Eastoe, 1954).

RESULTS

It was found necessary to control the pH of the buffers used in the region of pH 5·5–6·0, at least to within ± 0·01 pH unit, in order to give reasonably reproducible yields in the first peak. The shape of the curve for percentage of material eluted/pH (at constant loading; Fig. 3) shows why this is required. The consequent alteration of the buffer ionic strength during the adjustment of the pH, as will be seen later, has its own effect. For all elutions carried out in the present investigation the position of the first eluted peak remained unchanged, i.e. close to the fraction corresponding to the hold-up volume of the column. This implies negligible adsorption of the component constituting the first peak.

Once the conditions for reproducibility had been established it became possible to examine the remaining variables.

Temperature. Elutions carried out at a constant load and pH 5·52 on the small column in three separate experiments at 25°, 32·5° and 40° gave eluted peaks of 3·6, 3·3 and 3·4% respectively. Most of the subsequent experiments were carried out at 40° to avoid possible gelation taking place in the drop-counting mechanism of the fraction-collector.

Column loading. The results obtained at pH 5·25, 5·5 and 5·75 with various loadings are given in Table 1 and Fig. 2. Variation in loadings of 10–100 mg. on the small column at pH 5·25 does not produce any marked effect on the proportion of material eluted. At higher pH values, with gelatin no. 127, the effect of increased column loading becomes apparent. At pH 5·5 a marked increase in the percentage eluted occurs above a loading of 12 mg./small column. This behaviour is confirmed

by experiments carried out on large columns of 3 cm. diameter at pH 5·5. Thus columns 13·1, 13·7 and 11·9 cm. high, with loadings of 80, 400 and 1000 mg. respectively, equivalent to small column loadings of 12, 47 and 135 mg., gave proportions eluted of 1·0, 15·0 and 46·7 %. At pH 5·75 a column loading as small as 0·125 mg. still gives an increased proportion eluted, and thus the adsorption capacity of the column at this pH is very limited. The marked decrease in capacity of the resin on changing the pH from 5·25 to 5·50 to 5·75 may arise from changes both in ionization of the resin and of the gelatin in this region. The reproducibility of the results at pH 5·57 is low when different batches of buffer are used. It will be noted that the points representing experiments carried out with a single batch of buffer (*A*, Table 1) would fall on a smooth curve.

Table 1. *Variation of the proportion of gelatin eluted with column loading*

Columns were 14·0–15·0 cm. high × 1 cm. diameter.

pH	Load (mg. of air-dried material)		Concn. of gelatin solution added to column (mg./ml.)	Percentage eluted
5·25	10		5	1·4
	50		5	0·7
	100		5	0·6
5·50	5		5	1·1
	5		5	0·9
	10		5	1·1
	20		5	2·0
	20		10	2·7
	40		10	14·6
5·75	0·125	(A)*	1·25	18
	0·25	(A)	2·5	35
	0·5	(B)	2·5	39
	1·0	(C)	2·5	42
	1·0	(A)	2·5	50
	2·5	(C)	2·5	64
	2·5	(A)	2·5	70
	5	(A)	2·5	80
	8	(B)	2·5	73
	12·5	(A)	2·5	92

* Experiments with the same batch of buffer have the same letter.

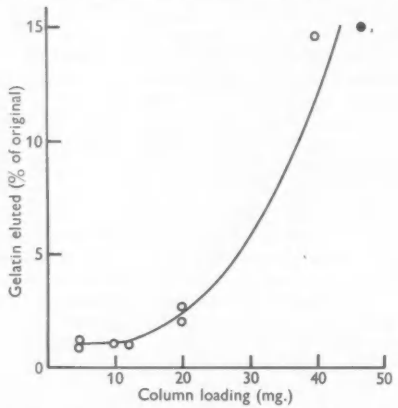

Fig. 2. Proportion of gelatin no. 127 eluted from small columns of Amberlite IRC-50 (XE-97), with McIlvaine buffers, pH 5·5, as a function of column loading (mg.). ○, Small columns; ●, with large column calculated to give equivalent small column load.

Resin batch. Results with small columns of XE-64 instead of XE-97 are shown in Table 2. At 3·1 % of material eluted the ratio of loadings XE-97:XE-64 was 8·8, and at 12·6 % eluted the ratio was 8·4. If the differences between the resins are only a function of surface area, then the mean-particle radii for the two resin batches would be in the ratio $\sqrt{(8{\cdot}6:1)}$, approx. 3:1. In view of the nominal mesh sizes of the resins (XE-97, 200–400 mesh; XE-64, 100–200 mesh) this difference in behaviour can be explained in terms of surface area/unit volume of resin.

pH. The effect of pH variation at a constant loading of 10 mg. of gelatin no. 127, with a small column, is shown in Fig. 3. In the range pH 3·0–5·0, 0·4–0·5 % of the original material was eluted. At pH 5·5 there is a small increase in the percentage eluted, and after pH 5·5 there is a more rapid increase followed by a sharp rise in the region of pH 5·55.The slight scatter of results on the steep portion of the curve arises from buffer variations.

Type of gelatin. The results with three gelatins differing in amide N and p*I* are given in Table 3. The pattern of behaviour of gelatins nos. 142 and 149, which have high p*I* values and high amide contents, is similar to that of no. 127 but takes place in a higher pH range. Although the p*I* of gelatin no. 149 is pH 9·4, it is probable that in the buffer used it is isoelectric in the region pH 5·0–6·0 (Veis, Anesey & Cohen, 1958; Jackson & Neuberger, 1957). This may explain why the pH for a given percentage eluted is not raised for gelatin no. 149 to a much greater extent.

Table 2. *Comparison of the behaviour of resins XE-97 and XE-64*

Load (mg.) on XE-64 (*Y*)	Percentage eluted from small column (*E*)	Load to produce (*E*) with small column of XE-97* (*X*)	(*X*)/(*Y*)
2·5	3·1	22	8·8
5·0	12·6	42	8·4

* Calculated from Fig. 2.

Molecular weight of gelatins. The behaviour of gelatins of differing molecular weights is shown in Table 4. The eluate from the high-molecular-weight fraction at pH 5·5 is significantly smaller than the amount with the unfractionated gelatin. The difference is probably too large to be ascribed solely to purification of the gelatin during fractionation. At pH 5·6 the low-molecular-weight fraction is much less firmly held, although poorer reproducibility at this pH limits the accuracy of the figures. Differences in molecular weight are not sufficient to account for the differences in behaviour shown by the three gelatins reported in Table 3.

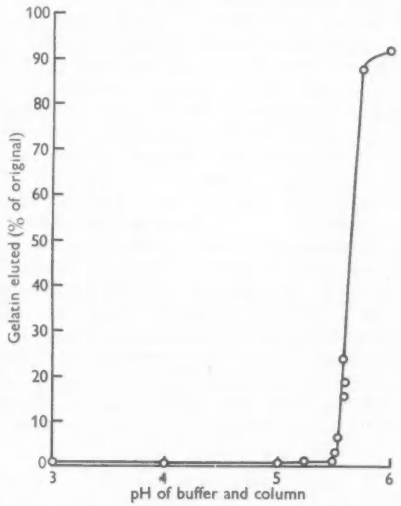

Fig. 3. Proportion of gelatin no. 127 eluted from small columns of Amberlite IRC-50 (XE-97), at a constant column load of 10 mg. of gelatin no. 127, as a function of the pH of the McIlvaine buffer used for elution.

Large-scale separations

Separation (a). Gelatin 127 was used on a column of XE-97 27 cm. high and 6 cm. diameter, at pH 5·0 and at 40°. The column was equilibrated with buffer containing a trace of phenylmercuric acetate as a preservative and the elution was carried out as described for small columns. A portion (720 ml.) of a 1 % solution of the gelatin in the buffer was placed on the column; this is equivalent to 100 mg. on the small column. Fractions of about 200 ml. were collected and two 1 ml. portions from each were used for colorimetric determinations of protein. As judged by the method of Lowry *et al.* (1951), 0·5 % of the original gelatin was eluted, i.e. 36 mg. from 7·2 g. of original gelatin. This was present in approx. 2 l. of buffer. The fractions containing the protein were dialysed in cellophan tubes for 3 days against tap water, and for a further 3 days against distilled water containing thymol, to remove buffer salts. The solution from the dialysis tubes was evaporated by boiling. As the volume of the solution reached about 200 ml. it became turbid and a white precipitate was formed. Evaporation was continued to

Table 3. *Effect of type of gelatin on the proportion eluted*

Column loading: 10 mg. added as 0·5 % solution.

Gelatin no.	p*I*	Amide N (g./100 g. of protein)	Percentage eluted		
			pH 5·5	pH 5·6	pH 6·0
127	4·95	0·16	1·1	19·4	93·0
142	7·8	0·50	1·3	2·2	64·0
149	9·4	0·62	0·9	1·1	30·7

Table 4. *Influence of gelatin molecular weight on percentage of material eluted*

Load: 10 mg. per small column.

Gelatin no.	M_w (mol.wt.)*	L.V.N.	Percentage eluted		
			pH 5·5	pH 5·6	
127/2/3/2	350 000	86·5	0·2	18·3 18·9	18·6
127	220 000†	64·0	1·0	15·7 18·6 24·0	19·4
127/4/3/4	120 000	55·5	1·5	45 56 60 79	60

* From L.V.N. values, as described under Materials. † Heterodisperse with respect to molecular weight.

50 ml. and the turbid solution was centrifuged. A white precipitate and a clear solution were obtained and these were analysed for amino acid composition separately.

The white precipitate was hydrolysed with 20% (w/w) HCl for 24 hr. at 100° in a sealed tube. The hydrolysate contained an appreciable amount of humin, admixed with what appeared to be extremely fine resin. The hydrolysate was filtered and diluted, and the acid was removed from portions in a vacuum desiccator over P_2O_5 and NaOH and analysed for amino acids.

The clear solution obtained above was made 20% (w/w) with respect to HCl and hydrolysed for 24 hr. in a sealed tube. Humin was again present, but in a much smaller quantity than that obtained above. A small amount of residue, probably resin, was also present. The analysis of amino acids was carried out as before.

The approximately 0·5% fraction, separated at pH 5·0, gave 10·36 mg. of amino acid residues (i.e. amino acids less 1 molecule of water) associated with the white precipitate, and 15·02 mg. in the clear solution. This corresponds to a total of 25·38 mg., i.e. 0·36% of the original gelatin, compared with 0·50% as judged by the method of Lowry *et al.* (1951). This difference arises since gelatin was used as the reference standard in the Lowry method. No difficulty was experienced for the larger percentages eluted since for these, as will be seen later, gelatin made up the bulk of the eluate protein. Results of the amino acid analyses are given in Table 5.

Before studying the amino acid composition of the larger amounts eluted, reported in Table 6, it is advisable to consider the limitations of the amino acid analyses. In the absence of either sample weight or figure for N, owing to lack of sufficient material, the sum of the residue weights was used to give the weight of material placed on the Dowex columns (Moore & Stein, 1951). This procedure means that any errors in one amino acid are also spread over all the amino acids and will be most apparent in the amino acids present in large quantities. Little importance can be attached to the figure for ammonia, as this has arisen in part from decomposition of mucoproteins and mucopolysaccharides and from other degradation reactions resulting from their presence. Some of the ammonia may have accumulated during isolation procedures. There were large amounts of mucopolysaccharides–proteins in the two fractions of

Table 5. *Amino acid composition of non-gelatin components*

A, Material from 0·36% fraction (associated with resin). *B*, Material from 0·36% fraction (free from resin). *C*, 0·36% fraction (combined analysis). *D*, Impurity prepared by Maron (1958). Approximately 0·3% in original gelatin: g. of amino acid/16 g. of N determined by quantitative paper chromatography. *E*, Amino acids plus hexosamines from 'osseomucoid' (Eastoe & Eastoe, 1954): g./115 g. of amino acid + hexosamines recovered.

Amino acid	*A*	*B*	*C*	*D*	*E*
	(g. of amino acid/100 g. of dry ash-free protein)				
Alanine	12·0	10·1	10·9	9·4	5·2
Glycine	5·9	9·1	7·9	13·1	3·8
Valine	6·9	7·0	6·9	6·6	6·4
Leucine	12·3	7·7	9·6	12·3	10·3
Isoleucine	5·2	3·7	4·3		5·1
Proline	4·4	5·1	4·9	7·0	6·0
Phenylalanine	5·5	4·7	5·0	3·5	4·1
Tyrosine	2·2	2·6	2·4	4·4	2·8
Tryptophan	Not determined				
Serine*	5·3	5·2	5·2	2·6	5·1
Threonine*	6·0	5·3	5·6	2·6	5·8
Cystine	Not determined			6·5	1·6
Methionine†	3·9	Trace	1·6	Trace	1·2
Arginine	8·0	6·4	7·1	7·8	5·5
Histidine	2·2	1·6	1·9	3·0	3·6
Lysine	6·1	6·1	6·1	5·2	6·0
Aspartic acid	10·5	13·6	12·3	10·5	13·7
Glutamic acid	14·3	16·1	15·3	15·9	16·5
Hydroxyproline	0·2	1·2	0·8	1·2	0
Hydroxylysine	0·8 (?)	Trace	0·3	—	0
Hexosamines	2·4	5·3	4·1	—	12·6
Amide‡	2·5	4·9	3·9	—	1·5

* Corrected for decomposition during hydrolysis.
† Sum of methionine and methionine sulphoxide peaks.
‡ Corrected for serine and threonine decomposition.

Table 6. *Amino acid composition of gelatin no.* 127 *and fraction*

A, Gelatin no. 127 (Eastoe, 1955). *B*, Purified gelatin no. 127 (Eastoe, 1955). *C*, 6·6 % fraction from gelatin no. 127. *D*, 6·6 % fraction corrected for 0·36 % fraction. *E*, 6·6 % fraction corrected for 0·36 % fraction on basis of relative hexosamines content.

Amino acid	Concn. (g./100 g. dry, ash-free protein)				
	A	*B*	*C*	*D*	*E*
Alanine	11·1	11·3	11·4	11·4	11·4
Glycine	27·2	27·6	23·0	23·9	25·3
Valine	2·82	2·63	3·0	2·8	2·0
Leucine	3·41	3·36	4·7	4·4	3·3
Isoleucine	1·68	1·70	2·1	2·0	1·5
Proline	16·3	16·5	14·2	14·7	15·7
Phenylalanine	2·24	2·23	2·8	2·7	2·4
Tyrosine	0·29	0·18	0·8	0·7	0·5
Tyrptophan	Not determined				
Serine*	4·11	4·15	3·6	3·6	3·3
Threonine*	2·23	2·27	2·6	3·6	3·3
Cystine	Not determined				
Methionine†	0·81	0·82	0·3	0·2	0·1
Arginine	9·1	8·9	7·5	7·5	7·6
Histidine	0·74	0·76	0·6	0·5	0·4
Lysine	4·70	4·70	6·0	6·0	5·9
Ornithine	Trace	Trace	Trace	Trace	Trace
Aspartic acid	6·6	6·6	6·5	6·0	5·5
Glutamic acid	11·1	11·4	12·7	12·6	12·2
Hydroxyproline	14·4	14·4	13·5	14·2	15·4
Hydroxylysine	1·07	1·04	1·0	1·0	1·0
Hexosamines	—	—	0·6	—	—
Amide‡	0·16	0·08	2·2	—	—

* Corrected for decomposition during hydrolysis.
† Sum of methionine and methionine sulphoxide peaks.
‡ Corrected for serine and threonine decomposition.

the 0·36 % material, eluted at pH 5·0, indicated by the humin formed and the hexosamines present.

The white precipitate obtained during the prehydrolysis evaporation stage and the humin-contaminated precipitate resulting from hydrolysis both had the appearance of resin. This must have arisen from a true solution, or from very finely dispersed colloidal solution, of resin in the eluting buffers, since the fractions obtained from the columns appeared perfectly clear. It has been found that, even after double pretreatments of the resin with acid and alkali, traces of the resin are still leached into the solution. These coagulate only after evaporation of the solution to a small volume or during acid hydrolysis of the solution.

Separation (*b*). The second large-scale experiment was carried out at pH 5·55 (the increased amount eluted being due to the increased pH) and 40° on a column of XE-97, 9·9 cm. high and 6 cm. in diameter. Gelatin no. 127 (50 ml. of 0·5 % solution) was used, equivalent to a load of 10 mg. on a small column. The experiment was carried out in the normal way and 6·6 % of the original material was eluted. In this experiment a precipitate did not form until the solution was hydrolysed. The hydrolysate contained a little humin together with a large amount of white precipitate, which was filtered off. The hydrolysate was analysed for amino acids as described earlier; the results are given in Table 6.

Separation (*c*). In the third experiment (the increased amount eluted was due to increased loading) a 400 mg. load of gelatin no. 127 was used on a column of XE-97, 13·7 cm. high and 9·9 cm. in diameter, at pH 5·5 and 40°; this is equivalent to a load of 47 mg. on a small column. Of the original material, 15 % was eluted. The fractions containing this material were dialysed, concentrated by evaporation and the protein was precipitated with a large excess of ethanol. It was then hydrolysed with 20 % (w/w) HCl and analysed for total N and hydroxyproline. The small amount of white precipitate appearing during hydrolysis was filtered off. The hydroxyproline N as a percentage of the total N was 7·3 %, compared with 8·5 % on the original gelatin. The decrease may be due either to the presence of non-collagenous proteins or to the preferential elution of gelatin molecules containing less hydroxyproline than the remainder. If it is assumed that the effect is due entirely to non-collagenous proteins and that the hydroxyproline content of the original high-grade gelatin represents approx. 100 % purity, then the eluted material represents 86 % of gelatin and 14 % of other proteins. That is, about 2 % of the original gelatin was eluted as other protein. This figure may be too high,

as some low-molecular-weight gelatin will have been lost during the ethanolic precipitation.

Separation (*d*). G. Russell (personal communication) has suggested that ionic strength is an important factor in the behaviour of gelatin on the Amberlite columns. This is to be expected from the extremely critical effect of pH on behaviour demonstrated in earlier experiments. The original McIlvaine buffers had an ionic strength in terms of citric acid molarity of 0·043 M for the pH 5·5 buffer; this was increased to 0·055 M at the same pH.

Gelatin no. 127 (83 mg.) was used on a column of XE-97, 13·4 cm. high and 3·0 cm. in diameter. This is equivalent to a loading of 10 mg. on a small column; 24·2 % of the gelatin was eluted. The fractions were dialysed, evaporated and hydrolysed, and the hydroxyproline N:total N ratio (as a percentage) was determined. This ratio was 7·3 %, which is approximately equivalent to 86 % of gelatin and 14 % of non-gelatin protein in the eluate. This is equivalent to 3·4 % of non-gelatin protein in the original gelatin. The inaccuracies in the various measurements suggest that this figure should be regarded only as indicative of a probable 2–4 % of non-gelatin proteins.

DISCUSSION

The amino acid composition of the peaks obtained in separations (*a*) to (*d*) reveal the main chemical features of the separation which the XE-97 resin can effect. The small peak (0·36 %) eluted from the lime-processed gelatin no. 127 has composition *C* (Table 5), made up from columns *A* and *B*. The hydroxyproline content of the eluted material indicates that not more than 5·5 % of gelatin was present. The formation of humin during hydrolysis, together with the presence of a substantial quantity of hexosamine, shows that the protein is probably present as mucoproteins. These are possibly partially degraded during treatment with alkali and originate from mucoproteins of the skin.

These mucoproteins are very similar in composition and behaviour to the mucoproteins ('osseomucoid') isolated from bone collagen by Eastoe & Eastoe (1954), and to the non-collagenous proteins obtained from calf skin by Bowes, Elliott & Moss (1958). The 'osseomucoid' was extracted by Eastoe & Eastoe from bone collagen with lime water. The composition after acid hydrolysis is shown in Table 5, column *E*. The three non-collagen-type protein fractions of Bowes *et al.* obtained from calf skin were: (*a*) citrate-soluble protein not precipitated by sodium chloride; (*b*) acetic acid-soluble protein not precipitated by sodium chloride; (*c*) dilute sodium hydroxide-soluble protein. The extracts showed the presence of hexosamines with the Elson & Morgan method (1933), thus indicating that they are either associated with polysaccharides or are in fact present as mucoproteins.

The 0·36 % material also shows a marked resemblance to the 0·30 % of protein isolated by Maron (1958) from a lime-processed gelatin by adsorption on active charcoal. The amino acid composition determined by him by quantitative paper chromatography is given in Table 5, column *D*. Maron does not record any investigation of hexosamines or mention humin formation during hydrolysis. However, the method of purification by precipitation at pH 4·0 which he describes indicates a similarity to the behaviour of mucoproteins in glues and some gelatins, which can be removed by centrifuging dilute aqueous solutions at pH 4·0 (author's unpublished observations).

The 0·36 % material isolated may also be identical with the heat-coagulable protein found in gelatins by Sheppard, Hudson & Houck (1931), for the mucoproteins isolated from glues and some gelatins, as described above, are denatured very rapidly by heat and then coagulate once the protective action of the gelatin is removed (author's unpublished observations).

Separations (*b*), (*c*) and (*d*), in which increased proportions were eluted by increasing the buffer pH (*b*), by increasing the column loading (*c*) and by increasing the buffer ionic strength (*d*), show that the increased proportion eluted from the columns under these conditions can be accounted for in terms of gelatin in addition to the small amount of non-gelatin proteins eluted in the lower pH ranges. Experiment (*b*) indicates that 0·36 % of the original gelatin has been eluted as non-gelatin protein along with about 6 % of gelatin. Experiments (*c*) and (*d*) indicate the level of non-gelatin protein in the original gelatin to be 2·0–3·5 %. These proportions are consistent with the work of Eastoe (1955), in which purification of gelatin by alcoholic coacervation indicates a level of impurity of this order.

The amino acid analysis of the 6·6 % fraction eluted at pH 5·55 is given in Table 6, column *C* and (i) corrected for the 0·36 % fraction, in column *D*, (ii) corrected for the 0·36 % fraction, the ratio of hexosamine contents being used as an index of contamination, in column *E*. The material corresponding to columns *C*, *D* and *E* of Table 6 closely resembles gelatin in composition, i.e. it shows high glycine, hydroxylysine, proline and hydroxyproline contents. It is not identical in composition with the parent gelatin (Table 6, column *A*) even after the above-mentioned corrections. There are two main reasons for this: (i) the limitations of the amino acid analyses already described; (ii) it has been shown that both the p*I* and the molecular

weight of the gelatin play a part in the adsorption on the resin; materials of lowest p*I* and lowest molecular weight are the least strongly adsorbed. If the separations are based on either of these factors it is possible that they may be associated with regions of the collagen chain differing slightly in composition from the remainder. There must come a stage, as the collagen chain is subdivided, where local differences in composition are sufficient to affect separation procedures.

The physicochemical experiments show that none of the variables examined shifts the position of the peak in relation to the volume of eluate, which occurs close to the hold-up volume of the column. The system corresponds to a single-plate column. The pattern of behaviour of acid-processed gelatins with change of pH is similar to that of lime-processed gelatins except that it takes place in a higher pH range.

The experiments discussed do not confirm the claim of Russell (1958) that 10–19% of a second, very different, component can be isolated from lime-processed gelatins with Amberlite IRC-50, XE-97 resins. It is not possible from the results described here to suggest the origin of the discrepancy.

SUMMARY

1. The behaviour of gelatins on columns of Amberlite resin IRC-50, XE-97 and XE-64 grades has been studied.

2. The proportion of gelatin eluted has been shown to depend on column loading, buffer pH and ionic strength, surface area of resin, type of gelatin and gelatin molecular weight.

3. A small proportion (0·36%) of a protein–polysaccharide complex, probably derived from mucoproteins, has been isolated from a lime-processed gelatin under certain conditions. When the proportion of the original material eluted is increased by raising the buffer pH, the ionic strength or the column load, the increase arises from gelatin passing through the column in addition to the small amount of other protein.

4. Acid-processed gelatins, which have higher amide contents and p*I* values than lime-processed gelatins, behave similarly to them but in a higher pH range.

5. The non-gelatin protein isolated is similar in amino acid composition to that already reported by Maron (1958).

6. The view that lime-processed gelatins contain 10–19% of a second, very different, component is not confirmed.

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APPENDIX

Notes on a Modification of the Neuman & Logan Method for the Determination of the Hydroxyproline

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The Neuman & Logan (1950) technique for the determination of hydroxyproline in protein hydrolysates consists of (i) oxidation of hydroxyproline with hydrogen peroxide in the presence of alkaline copper sulphate, (ii) destruction of excess of peroxide by heat and (iii) reaction of the oxidation product with *p*-dimethylaminobenzaldehyde by heating in the presence of dilute sulphuric acid to produce a red colour, the intensity of which is compared with a standard. The original method has been modified by a number of workers (Baker, Lampitt & Brown, 1953; Martin & Axelrod, 1953; Wierbicki & Deatherage, 1954; and Miyada & Tappel, 1956) in an attempt to improve (*a*) the removal of the hydrogen peroxide, which, if undestroyed, leads to low and variable colour yields, (*b*) the reproducibility amongst replicates and (*c*) the colour yield. The present modification has, in our Laboratories, given a greater reproducibility amongst replicates and colour yields reproducible over a prolonged period. In the modification to be described, stages (i) and (ii) of the original method are combined. The oxidation is carried out at 40° and is completed before the total destruction of the excess of peroxide has occurred. For ease of reference the parts of the original Neuman & Logan method used are described with the modification.

REAGENTS

0·05 M-*Copper sulphate* (*A.R.*) *in water*. This is the concentration recommended by Baker *et al.* (1953), and results in higher colour yields than the 0·01 M concentration recommended by Neuman & Logan (1950).

2·5 N (*approx.*)-*Sodium hydroxide*. Sodium hydroxide (100 g. of pellets; A.R.) was made up to 1 l. with water.

6% (*approx.*) *Hydrogen peroxide*. This was made by diluting A.R. 30% (w/v) hydrogen peroxide. The diluted solution is not reliable for more than a day and it is advisable to make up a fresh solution immediately before use. The parent solution is stable over a long period, but can be checked by the method of Vogel (1957).

3 N (*approx.*)-*Sulphuric acid*. The solution contained 83 ml. of A.R. conc. H_2SO_4/l.

5% p-*Dimethylaminobenzaldehyde*. The reagent (May and Baker Ltd.) dissolved in redistilled propan-1-ol (fraction 95·8–98·0°) has been found satisfactory. This solution should be stored in the dark.

Standard L-*hydroxyproline solutions*. Hydroxyproline (0·05 g.) was dissolved in water and diluted to about 400 ml. with water, 20 ml. of conc. HCl was added (J.E. Eastoe, personal communication) and the solution made up to 500 ml. with water. The acid prevents microbiological destruction; the solution will keep only for a short period if the HCl is omitted. The 100 μg./ml. solution is diluted to give concentrations of 5, 10 and 15 μg. of hydroxyproline/ml. The amino acid samples supplied by British Drug Houses Ltd. and L. Light and Co. Ltd. were found to be pure and dry. Drying at 105° for 2 hr. is, however, a safeguard against moisture.

PROCEDURE

The solution under test should contain preferably between 5 and 15 μg. of hydroxyproline/ml. For a single determination 13 1 in. × 6 in. rimless Pyrex test tubes are required. In each of tubes 1–3 is placed 1 ml. of 5 μg. standard, in tubes 4–6 1 ml. of 10 μg. standard, in tubes 7–9 1 ml. of 15 μg. standard, in tubes 10–12 1 ml. of the test solution and in tube 13 1 ml. of water. Into each tube is placed 1 ml. of 0·05 M-$CuSO_4$, followed by 1 ml. of 2·5 N-NaOH, and the tube contents are each mixed by gentle swirling of the liquid. The tubes are placed in a water bath at 40°. When the contents have reached 40° (3–5 min.), 1 ml. of 6% hydrogen peroxide is added, which is immediately mixed by swirling the contents of a tube before the addition is made to the next tube. During the swirling of the contents no solution should remain on the wall of the tube above the level reached by the water in the bath, otherwise the destruction of the peroxide is incomplete. The tubes are left in the bath for 10 min., but are occasionally removed from the bath and the contents swirled. The tubes are cooled with tap water, then 4 ml. of 3 N-H_2SO_4 and 2 ml. of 5% *p*-dimethylaminobenzaldehyde solution are added, the contents of the tubes being mixed by swirling after each addition. Caps (Oxoid) are placed on the tubes, which are kept in a water bath at 70° for 16 min.; the solutions are then cooled, mixed and their extinctions measured against the blank solution at a wavelength of 555 mμ in 4 cm. cells.

The average reading for each set of tubes is used in the calculation. In order to minimize the effect

of a slight negative deviation from Beer's law it is advisable to draw straight lines between each pair of standard values rather than a single line through the whole set.

RESULTS

Fig. 1 shows the variation of extinction with the temperature of the first bath with the modified technique. The values obtained for an average run by the original method are included for comparison. At first bath temperatures below 35° the colour yields are not reproducible and below 25° the normal reddish pink is sometimes replaced by an orange oxidation product, resulting from the presence of undestroyed hydrogen peroxide. The points below 25° are the average of a number of determinations where the colour did appear and are included to give an idea of the order of the decreased colour yield.

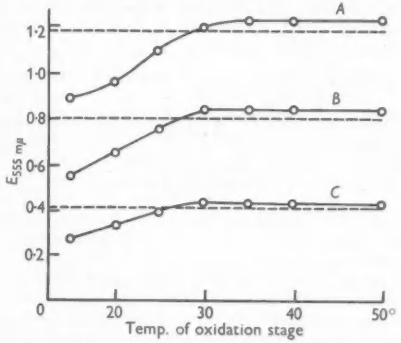

Fig. 1. Temperature of oxidation versus colour yield: *A*, 15 μg. of hydroxyproline/ml.; *B*, 10 μg. of hydroxyproline/ml.; *C*, 5 μg. of hydroxyproline/ml. Broken lines refer to average values obtained by the original Neuman & Logan method at the concentrations given above. A 4 cm. cell was used.

The scatter amongst replicates is less with the modified method; for example, carrying out both methods with the same solutions gave standard deviations, amongst replicates for the three standards and unknown, of 0·74 and 0·49% by the original and modified methods respectively. The modified method has often yielded standard deviations amongst replicates of the order 0·1–0·2%, an order of accuracy which was not achieved with the original method over a considerable period. The reproducibility of the colour yield from one series to another has been very high over a prolonged period. The colour yields are represented by the plateaus in Fig. 1, and the reproducibility of the method is such that it serves as a guide to reagent quality. A decrease in colour yield by 4% or more from the values shown indicates inferior or decomposing reagents; or, if acid is omitted from the hydroxyproline solutions, that microbiological decomposition of the amino acid is taking place.

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Spectrophotometric Studies on the Combination of Formaldehyde with Tetrahydropteroylglutamic Acid and Other Hydropteridines

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A previous paper (Blakley, 1959) described studies on the reaction of formaldehyde with tetrahydropteroylglutamic acid and other hydropteridines, which were undertaken to elucidate the coenzyme function of tetrahydropteroylglutamate in the synthesis of serine from glycine and formaldehyde.

It was found that formaldehyde reacted with all the hydropteridines studied, but the complexes formed differed greatly in the extent to which they were dissociated. Formaldehyde was very firmly bound by tetrahydropteroylglutamate, the dissociation constant for the complex being at least 100 times lower than those for the complexes formed by simple hydropteridines. Since N^5-formyltetrahydropteroylglutamate (leucovorin) and N^{10}-formyltetrahydropteroylglutamate also formed

complexes with relatively high dissociation constants, it was concluded that tetrahydropteroylglutamate reacts reversibly with formaldehyde to form N^5N^{10}-methylenetetrahydropteroylglutamate, whereas all other hydropteridines studied (except possibly tetrahydropteroic acid) formed hydroxymethyl derivatives. The point of attachment of the hydroxymethyl group in these derivatives was indicated by the fact that the N^5-formyl derivative of a simple hydropteridine formed a complex with a dissociation constant considerably higher than that of the complex from the parent hydropteridine. It was concluded therefore that simple hydropteridines form highly dissociated N^5-hydroxymethyl derivatives.

In this previous investigation the values of the dissociation constants were obtained from determinations of the free formaldehyde in equilibrium with known concentrations of hydropteridines. A limitation was placed on the accuracy of the results for tetrahydropteroylglutamate and related compounds by the fact that large blanks were obtained in the colorimetric determination of formaldehyde when these hydropteridines were present. Since only very low concentrations of formaldehyde existed in equilibrium with tetrahydropteroylglutamate, the relative error was highest in this case. As this was the reaction of primary interest, another approach seemed necessary. Spectrophotometric studies were therefore undertaken to obtain further information about the combination of formaldehyde with tetrahydropteroylglutamate and for comparative purposes the reaction of formaldehyde with other hydropteridines was also studied. Clear evidence has been obtained for the reversibility of the reaction between tetrahydropteroylglutamate and formaldehyde and a more accurate estimate obtained for the dissociation constant of the product. In addition, more data have been obtained on the reaction of formaldehyde with other pteridines and hydropteridines which shed light on the compounds formed.

EXPERIMENTAL

Materials

All pteridine derivatives were prepared as previously described (Blakley, 1959). The following additional compounds were prepared as indicated: 2-hydroxypyrimidine, Brown (1950); 2-methoxypyrimidine, Brown & Short (1953).

Methods

Spectrophotometric readings. Manually operated instruments (Shimadzu and Unicam) were used in the original experiments and the results were checked with a Beckman DK 2 ratio-recording spectrophotometer. The reference cell contained all the reagents present in the sample cell, except the pteridine or pyrimidine under study. During kinetic studies exactly the same additions of reagents were made to the reference cell as to the sample cell.

Measurement of pH. A glass electrode, with 5 mM-sodium borate to provide the reference pH, was used. Potassium phosphate buffer was prepared according to the data of Green (1933). The concentrations of phosphate and *N*-ethylmorpholine buffers given refer to anion and cation concentrations respectively.

Calculation of dissociation constants. In calculating the dissociation constants of the complexes formed by formaldehyde with various bases, the assumption was made that each complex was formed by reaction of equimolecular proportions of base and formaldehyde thus:

$$\text{Base} + \text{H·CHO} \rightleftharpoons (\text{Base,H·CHO})\ \text{Complex}. \qquad (1)$$

If the initial concentrations of base and formaldehyde, $[B]_i$ and $[H\cdot CHO]_i$ respectively, were known, then the equilibrium concentrations of these compounds, $[B]_{equ}$ and $[H\cdot CHO]_{equ}$ respectively, could be calculated from the equilibrium concentration of the complex, $[complex]_{equ}$. This follows from the fact that the decrease in concentration of base and of formaldehyde is equal to the equilibrium concentration of complex,

$$[\text{Complex}]_{equ} = [B]_i - [B]_{equ} = [H\cdot CHO]_i - [H\cdot CHO]_{equ}. \qquad (2)$$

The dissociation constant, K, could then be calculated from the relationship

$$K = \frac{[B]_{equ}[H\cdot CHO]_{equ}}{[\text{Complex}]_{equ}}. \qquad (3)$$

The equilibrium concentration of the complex was determined spectrophotometrically as follows. The extinction of the pure base at a concentration $[B]_i$ was determined at a wavelength where complex formation caused a large change in extinction. Let E_B be the determined extinction of the pure base at concentration $[B]_i$ at this wavelength. Base at a concentration $[B]_i$ was then equilibrated with a high concentration of formaldehyde. Assuming complete conversion of the base into complex, the extinction of the solution equalled E_C, the extinction of the complex at a concentration equal to $[B]_i$. If now base at a concentration $[B]_i$ was allowed to equilibrate with a low concentration of formaldehyde $[H\cdot CHO]_i$, the measured extinction E, due to the equilibrium mixture of base and complex, was intermediate between E_B and E_C. The proportion of complex in the mixture was given by the ratio $(E-E_B)/(E_C-E_B)$ so that the equilibrium concentration of complex is given by

$$[\text{Complex}]_{equ} = \frac{E-E_B}{E_C-E_B}[B]_i.$$

Substitution of this value in equations (2) and (3) enabled calculation of the dissociation constant. If the concentration of formaldehyde employed in the determination of E_C was not high enough, then some free base remained in the solution, leading to an error in E_C which resulted in a low value for $E_C - E_B$. On the other hand, care was necessary that the level of formaldehyde used was not so high that secondary reactions occurred leading to extraneous spectrum changes. With tetrahydropteroylglutamate the concentrations of formaldehyde used in determining E_C was 10 mM.

An alternative procedure was used in determining pK spectrophotometrically: values of the extinction at the selected wavelength were plotted against pH. From the graph the pH value was obtained at which the extinction was midway between those of the two ionic species. This pH was taken as equal to the pK.

RESULTS

4-Hydroxy-6-methyltetrahydropteridine. The spectrum of 4-hydroxy-6-methyltetrahydropteridine at pH 7·2 is shown in Fig. 1. Absorption maxima occurred at 220 and 288 mμ and a minimum of 245 mμ. Although this compound was relatively stable in neutral solution the spectrum changed significantly over a period of hours. The shape of the absorption curve did not change but the extinction slowly decreased, indicating the formation of non-absorbing products.

On addition of formaldehyde to give a concentration of 25 mM the spectrum was shifted towards longer wavelengths with a slight reduction in the extinction maximum (Fig. 1). Rate studies showed that this reaction was 50% complete in about 2·5 min. so that its rate was about the same as that for the combination of tetrahydropteroylglutamic acid (tetrahydroPGA) and formaldehyde (cf. Blakley, 1959). More detailed studies showed that at high concentrations of formaldehyde the 220 mμ maximum is shifted to longer wavelengths with a simultaneous increase in the extinction maximum (Fig. 1). Moreover, the 288 mμ peak, which is shifted to 292 mμ by 25 mM-formaldehyde, shifts back to shorter wavelength at higher formaldehyde concentrations. In addition, the absence of an isosbestic point in this region of the spectrum (Fig. 1) indicates the formation of more than one product. An approximate calculation of the dissociation constant of the final product from the extinctions obtained at 235 mμ (see Methods section) indicated that the constant was about 0·2 M. This is much higher than the value of 0·03 M obtained for the N^5-hydroxymethyl derivative by chemical analysis (Blakley, 1959), so that the changes in spectrum at the higher concentrations of formaldehyde corresponded to the formation of another compound. The spectrum change corresponding to the secondary reaction made it impossible to obtain a value for the dissociation constant of the N^5-hydroxymethyl derivative by the spectrophotometric method.

N^5-Formyl-4-hydroxy-6-methyltetrahydropteridine

Since the spectrum changes obtained when 4-hydroxy-6-methyltetrahydropteridine was treated with high concentrations of formaldehyde appeared due to the combination of the latter at some site

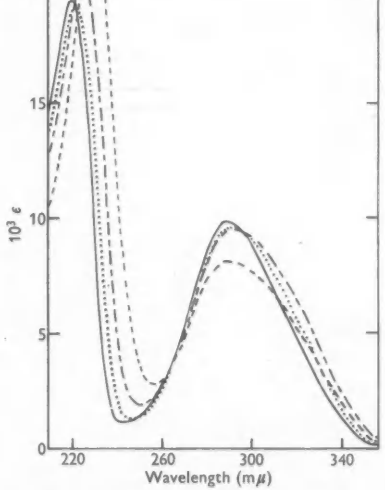


Fig. 1. Absorption spectra of 4-hydroxy-6-methyltetrahydropteridine (0·04 mM) in 0·1 M-potassium phosphate buffer, pH 7·2. —, No formaldehyde; . . ., in the presence of 25 mM-formaldehyde; — · —, in the presence of 0·15 M-formaldehyde; - - -, in the presence of 5·0 M-formaldehyde.

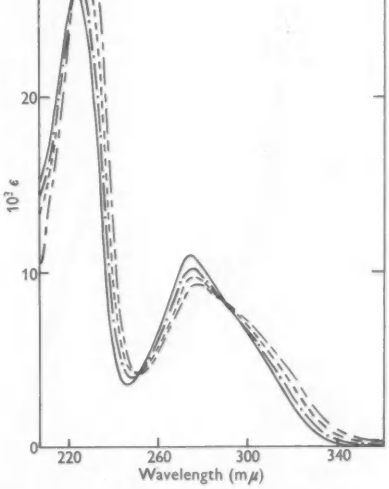
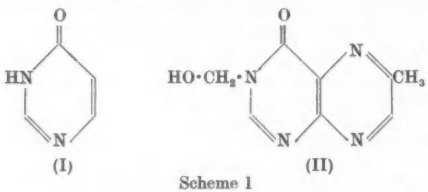


Fig. 2. Absorption spectra of N^5-formyl-4-hydroxy-6-methyltetrahydropteridine (0·03 mM) in 0·1 M-potassium phosphate buffer, pH 7·2. —, No formaldehyde; — · —, in the presence of 0·15 M-formaldehyde; - - -, in the presence of 0·75 M-formaldehyde; — - —, in the presence of 5·0 M-formaldehyde.

other than N^5 of the hydropteridine, it seemed probable that N^5-formyl-4-hydroxy-6-methyltetrahydropteridine would show spectrum changes in the presence of formaldehyde similar to the secondary change shown by the parent compound, and this was in fact found to be the case (Fig. 2). When the spectra obtained at various formaldehyde concentrations were superimposed isosbestic points were obtained at 221, 253 and 292 mμ, indicating the formation of only one product. The close agreement between the dissociation constant (0·3 M) calculated from the spectrum change for this compound, and that given above for the product formed by the parent 4-hydroxy-6-methyltetrahydropteridine at high formaldehyde concentrations, suggests that formaldehyde was reacting at the same site in the two molecules.

In the formyl derivative there are three possible sites for the reaction with formaldehyde; i.e. N^8 in the hydrogenated ring and N^1 and N^3 in the pyrimidine ring. The spectrum of 4-hydroxy-6-methylpteridine was investigated in an attempt to resolve this problem and was found also to undergo a shift in the presence of formaldehyde (Fig. 3), corresponding to formation of a product with dissociation constant approximately 0·3 M. Pteroylglutamic acid, 2-deaminopteroylglutamic acid and N^5-formyltetrahydropteroylglutamic acid (leucovorin) displayed similar changes in spectra in the presence of formaldehyde at high concentrations, forming complexes with approximate dissociation constants of 0·3, 0·4 and 0·4 M respectively. These results suggest that formaldehyde was reacting at a site in the pyrimidine ring in all the above-mentioned compounds.

Reaction of formaldehyde with pyrimidines. Since 4-hydroxypyrimidine exists mainly in the lactam (ketonic) form (scheme 1; I) at neutral pH (Brown, Hoerger & Mason, 1955), it seemed possible that similar tautomerism in 4-hydroxy-6-methylpteridine might allow reaction of formaldehyde at N^3 to give an N^3-hydroxymethyl derivative (scheme 1; II). To test this hypothesis the effect of formaldehyde on 2-hydroxypyrimidine and on 2-methoxypyrimidine was investigated. At high concentrations of formaldehyde the extinction maximum of 2-hydroxypyrimidine was shifted to longer wavelengths and slightly increased (Fig. 4) in a manner exactly analogous to that described above for various 4-hydroxypteridines, but the spectrum of



Scheme 1

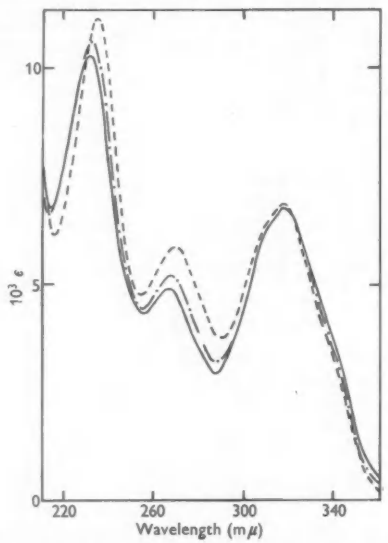


Fig. 3. Absorption spectra of 4-hydroxy-6-methylpteridine (0·075 mM) in 0·1 M-potassium phosphate buffer, pH 7·2. —, No formaldehyde; —·—, in the presence of 0·15 M-formaldehyde; - - - -, in the presence of 5·0 M-formaldehyde.

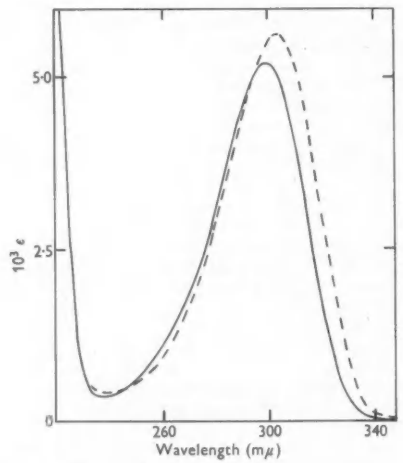


Fig. 4. Absorption spectra of 2-hydroxypyrimidine (0·1 mM) in 0·1 M-potassium phosphate, pH 7·2. —, No formaldehyde; - - - -, in the presence of 5·0 M-formaldehyde.

2-methoxypyrimidine at pH 7·2 ($\lambda_{max.}$ 264 mμ, $\epsilon_{max.}$ 4700) was unchanged by 5M-formaldehyde. The spectrum change for 2-hydroxypyrimidine corresponded to a product with dissociation constant of 0·4M. These results strongly suggest formation of N^3-hydroxymethyl-2-oxopyrimidine from 2-hydroxypyrimidine by the action of formaldehyde in high concentrations.

Spectra of tetrahydroquinoxaline and 2-chloro-4-methyl-N^8-benzyltetrahydropteridine. In these two compounds there is no possibility of a reaction of formaldehyde with pyrimidine ring nitrogen. Tetrahydroquinoxaline has a benzene ring instead of a pyrimidine ring and in 2-chloro-4-methyl-N^8-benzyltetrahydropteridine no hydrogen is able to migrate to N^3. With the former compound the addition of 25 mM-formaldehyde to the solution at pH 7·2 caused the spectrum to be displaced towards longer wavelength, together with a decrease in the maximum extinction. At high formaldehyde concentrations, however, the maximum extinction was greater than for the untreated compound as well as being displaced towards longer wavelengths (Fig. 5). This suggests the formation of two derivatives, presumably N^5-hydroxymethyl and N^5N^8-bis-(hydroxymethyl). The dissociation constant calculated from the extinction at 230 mμ at various formaldehyde concentrations was about 0·08M, which is about ten times higher than that previously assigned to the N^5-hydroxymethyl compound (Blakley, 1959) and probably refers to the dissociation of the N^8-hydroxymethyl group.

Formaldehyde decreased the extinction maximum of 2-chloro-4-methyl-N^8-benzyltetrahydropteridine as shown in Fig. 6. From the extinction figures at 310 mμ a value of 0·5M was obtained for the dissociation constant, which is rather high compared with the value (0·29M) previously obtained (Blakley, 1959).

Spectrophotometric determination of pK of 4-hydroxy-6-methyltetrahydropteridine. If the N^5-amine grouping of 4-hydroxy-6-methyltetrahydropteridine has a basic pK near the pH at which measurements of formaldehyde-binding have been made (pH 7·2), the measured binding of formaldehyde would be unduly low because of the existence of some of the hydropteridine in the protonated form which may not combine with formaldehyde. To investigate this possibility a spectroscopic investigation of the pK of this hydropteridine was made. Titration had already shown that the pK of the 4-hydroxy group was 10·2 (Blakley, 1959). The spectrum did not change significantly between this pH and pH 5·0; but below pH 5·0 the absorption maximum shifted from 288 to 260 mμ. By plotting

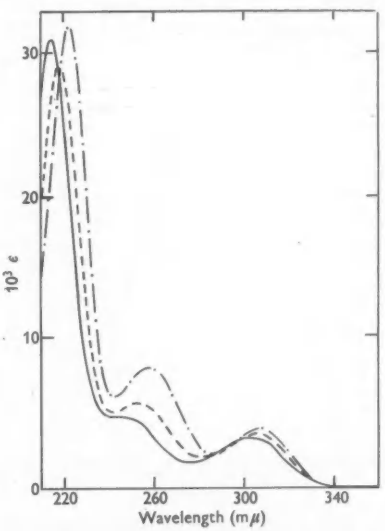


Fig. 5. Absorption spectra of tetrahydroquinoxaline (0·025 mM) in 0·1M-potassium phosphate, pH 7·2. —, No formaldehyde; - - - -, in the presence of 25 mM-formaldehyde; —·—, in the presence of 5·0M-formaldehyde.

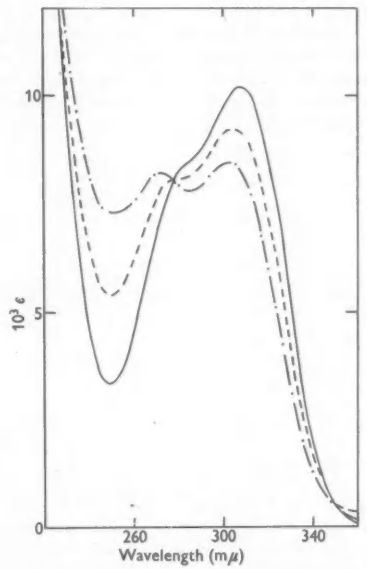


Fig. 6. Absorption spectra of N^8-benzyl-2-chloro-4-methyltetrahydropteridine (0·075 mM) in 0·1M-potassium phosphate, pH 7·2. —, No formaldehyde; - - - -, in the presence of 0·75M-formaldehyde; —·—, in the presence of 5·0M-formaldehyde.

the extinction at 260 mμ and at 290 mμ against pH (see Methods section), a value of 4·1 was obtained for the pK. Even if this pK were attributed to the N^5-amine group (rather than to the N^8-amine group to which it more probably belongs; see Discussion), protonation at pH 7·0 would be negligible.

Reaction of formaldehyde with tetrahydropteroylglutamic acid

In order to study the effect of formaldehyde on tetrahydroPGA, the instability of the latter had to be overcome. The spectrum of a solution of tetrahydroPGA at neutral pH changed rapidly with a shift of the extinction maximum from 298 to about 274 mμ. This change, which indicated oxidative degradation of tetrahydroPGA, could be retarded by the addition of various stabilizing substances to the solution. Chelating agents such as ethylenediaminetetra-acetic acid (EDTA) and 8-hydroxyquinoline-5-sulphonic acid caused some stabilization, as seen in Fig. 7, but reducing agents such as thiols were rather more effective. By far the most efficient stabilizing compound was 2:3-dimercaptopropan-1-ol (BAL) which, in mM concentration, prevented oxidative degradation of tetrahydroPGA at neutral pH for several hours. In the presence of 0·5 mM-BAL only slight oxidation occurred over a period of 25 min. (Fig. 7).

The spectrum change shown by neutral solutions of tetrahydroPGA was also retarded by the presence of formaldehyde. Addition of formaldehyde to give a final concentration of 0·1 mM caused a significant decrease in the rate at which 0·03 mM-tetrahydroPGA was degraded, and in the presence of mM-formaldehyde degradation was almost completely arrested (Fig. 8).

This stabilizing effect of formaldehyde on tetrahydroPGA is interpreted as due to combination of formaldehyde with tetrahydroPGA to form a product which is not, or is very slowly, attacked by oxygen. Since oxidative degradation eventually proceeds to completion even when a large excess of formaldehyde is present, the formation of the oxygen-stable complex must be a reversible reaction. To obtain further data on tetrahydroPGA–formaldehyde combination the reaction has been studied in the presence of mM-BAL. At pH 7·2 addition of formaldehyde to such a stabilized solution of tetrahydroPGA causes an increase in the maximum extinction and a slight shift to shorter wavelength (Fig. 9). The extent of the spectrum shift varies with formaldehyde concentration, indicating a reversible reaction, but is complete in 10 mM-formaldehyde. In the absence of formaldehyde $\lambda_{max.}$ is 298 mμ and $\epsilon_{max.}$ is 28 400, whereas in the presence of 10 mM-formaldehyde $\lambda_{max.}$ is 294 mμ and $\epsilon_{max.}$ is 32 000. Isosbestic points may be seen to occur at 249 and 270 mμ, an indication that only a single product is formed in significant amounts. In the presence of 10 mM-formaldehyde the same spectrum is obtained whether BAL is present or absent.

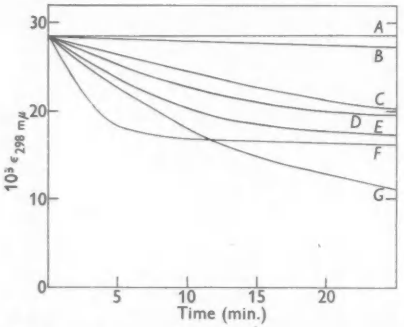


Fig. 7. Rate of change of the spectrum of tetrahydroPGA (0·03 mM) in 0·1 M-potassium phosphate buffer, pH 7·2. *A*, In the presence of mM-BAL; *B*, in the presence of 0·5 mM-BAL; *C*, in the presence of 2 mM-2-mercaptoacetic acid; *D*, in the presence of 5 mM-2-mercaptoethanol; *E*, in the presence of mM-2-mercaptoethanol; *F*, no addition; *G*, in the presence of 0·2 mM-8-hydroxyquinoline-5-sulphonic acid.

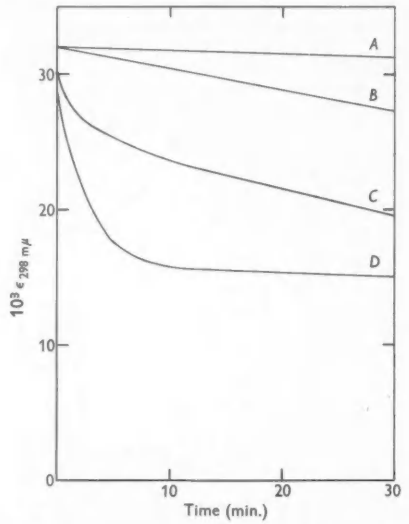


Fig. 8. Rate of change of the spectrum of tetrahydroPGA (0·03 mM) in 0·1 M-potassium phosphate buffer, pH 7·2. *A*, in the presence of 10 mM-formaldehyde; *B*, in the presence of mM-formaldehyde; *C*, in the presence of 0·1 mM-formaldehyde; *D*, no formaldehyde.

The rate of the spectrum change accompanying reaction of formaldehyde with tetrahydroPGA is shown in Fig. 10. In the experiment in which both reactants were at a concentration of 0·4 mM the reaction was 40 % complete in the first minute. This reaction rate is similar to that observed by measuring formaldehyde disappearance from the reaction mixture (Blakley, 1959).

At alkaline pH it was more difficult to observe the formaldehyde–tetrahydroPGA reaction spectrophotometrically, although previous evidence had indicated that the reaction proceeded even at pH 13·0 (Blakley, 1959). TetrahydroPGA, in the presence of mM-BAL, showed a considerable spectrum change over a period of 30 min. due to degradation, $\lambda_{max.}$ changing from 292 to 285 mμ, which made observations on the formaldehyde reaction difficult. Owing to the absorption by the BAL, readings could not be made below 270 mμ. The addition of formaldehyde (final concentration 10 mM) to a freshly prepared solution of 0·03 mM-tetrahydroPGA in mM-BAL and 0·01 N-sodium hydroxide resulted in only a very slight increase in the maximum extinction, together with a broadening of the absorption peak (Fig. 11). Observations on the formaldehyde–tetrahydroPGA reaction at low pH were extremely difficult because sulphydryl reagents no longer prevented oxidative degradation of tetrahydroPGA under these conditions, and other reducing agents which were tried were unsatisfactory.

Further evidence for the reversibility of the tetrahydroPGA–formaldehyde reaction. Data have been presented above which indicate that the reaction of tetrahydroPGA and formaldehyde is reversible. As a further test of reversibility the reaction between tetrahydroPGA (0·05 mM) and formaldehyde (0·15 mM) in the presence of mM-BAL was allowed to come to equilibrium, as shown by the recording of the extinction at 300 mμ (Fig. 12). A small volume of bisulphite solution was then added to both the reference and sample cells and the mixture rapidly stirred. The extinction at 300 mμ rapidly fell until a new equilibrium level had been reached. A final concentration of 0·33 mM-bisulphite lowered the extinction at 300 mμ to a value approaching that for tetrahydroPGA. The absorption spectrum was similar to that of 0·05 mM-tetrahydroPGA. Addition of this amount of bisulphite to tetrahydroPGA in the presence of mM-BAL caused no change in spectrum.

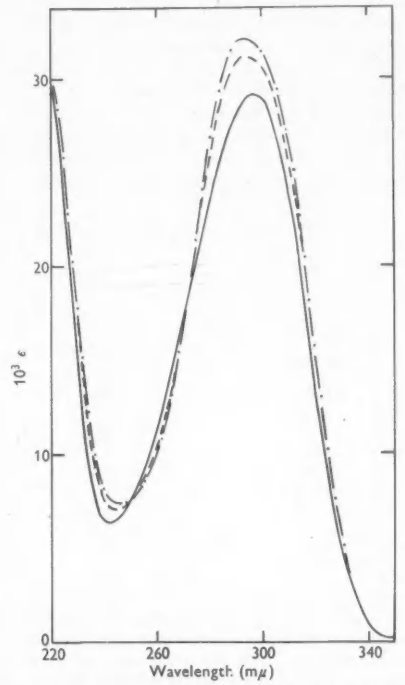


Fig. 9. Absorption spectra of tetrahydroPGA (0·03 mM) in 0·1 M-potassium phosphate buffer, pH 7·2, containing mM-BAL. ——, No formaldehyde; - - -, in the presence of 0·067 mM-formaldehyde; —·—, in the presence of 10 mM-formaldehyde.

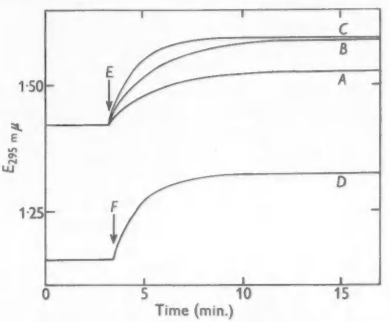


Fig. 10. Rate of reaction of tetrahydroPGA with formaldehyde. Curves *A*, *B* and *C*: rate of change of extinction, at 20° with 1 cm. light path, of 0·05 mM-tetrahydroPGA in 0·1 M-potassium phosphate buffer, pH 7·2, containing mM-BAL. Formaldehyde was added at point *E* to give the following final concentrations: curve *A*, 0·05 mM-formaldehyde; curve *B*, 0·1 mM-formaldehyde; curve *C*, 0·2 mM-formaldehyde. Curve *D*: rate of change of extinction at 20°, with 0·1 cm. light path, of 0·4 mM-tetrahydroPGA in 0·1 M-potassium phosphate buffer, pH 7·2, containing mM-BAL. At point *F* formaldehyde added to give a final concentration of 0·4 mM. Extinction was corrected for dilution by added formaldehyde.

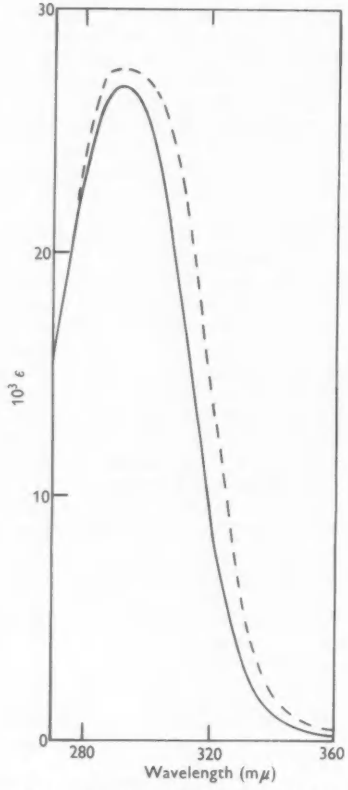


Fig. 11. Absorption spectra of tetrahydroPGA (0·03 mM) in 0·01 N-NaOH containing mM-BAL. ——, No formaldehyde; - - - -, 10 mM-formaldehyde.

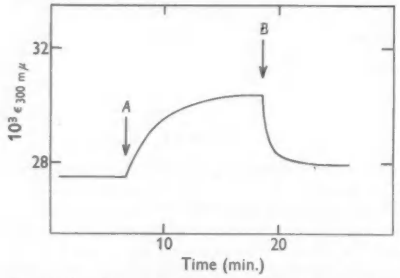


Fig. 12. Effect of formaldehyde and subsequent addition of bisulphite on the spectrum of tetrahydroPGA. Tetrahydro PGA (0·05 mM) in 0·1 M-potassium phosphate buffer, pH 7·2, containing mM-BAL was treated at 20° with formaldehyde (final concn. 0·15 mM) at point *A*. At point *B* bisulphite (final concn. 0·33 mM) was added. Curve is corrected for dilution effect of additions.

The value of the dissociation constant, defined by

$$\frac{[\text{TetrahydroPGA}]\,[\text{H·CHO}]}{[\text{Complex}]}$$

for the product of the reaction between tetrahydroPGA and formaldehyde was obtained from series of determinations of extinction at the wavelengths 295, 298 or 300 mμ when 0·05 mM-tetrahydroPGA was allowed to equilibrate with formaldehyde at concentrations between 0·05 and 0·15 mM (see Methods section). In an early experiment, with a manual spectrophotometer, the value of the dissociation constant obtained was $4{\cdot}79 \pm 0{\cdot}30 \times 10^{-5}$ M from reaction in the presence of mM-BAL and potassium phosphate buffer, pH 7·2 (0·1 M), at 20°. Seven more recent determinations made with the Beckman recording spectrophotometer have given a value of $3{\cdot}12 \pm 0{\cdot}19 \times 10^{-5}$ M. The same result is obtained in the presence of 0·15 mM-BAL.

Stability of the product of the reaction of formaldehyde and tetrahydroPGA. At equilibrium 0·1 mM-formaldehyde will convert 0·03 mM-tetrahydroPGA almost entirely into the complex, which is believed to be N^5N^{10}-methylenetetrahydropteroylglutamic acid. The rate of subsequent change of the spectrum will therefore indicate the stability of methylenetetrahydropteroylglutamic acid. It may be seen from Fig. 13 that in 0·1 M-*N*-ethylmorpholine buffer, pH 7·2, or in 0·01 M-potassium phosphate buffer, pH 7·2, the decomposition of the tetrahydroPGA occurred so rapidly that it was virtually com-

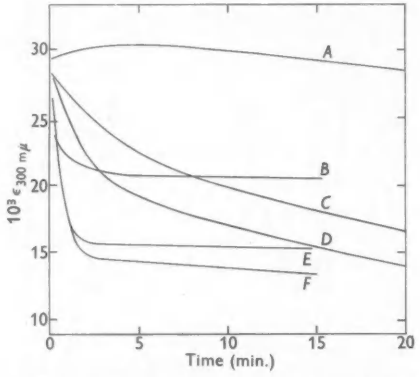


Fig. 13. Rate of change of the spectrum of tetrahydroPGA (0·03 mM) at 20° in the presence of 0·1 mM-formaldehyde and the following additional components, added at zero time: curve *A*, 0·1 M-*N*-ethylmorpholine, pH 7·2, and 0·1 mM-8-hydroxyquinoline-5-sulphonic acid; curve *B*, 0·01 N-NaOH; curve *C*, 0·1 M-*N*-ethylmorpholine, pH 7·2, and 0·01 M-EDTA, pH 7·2; curve *D*, 0·1 M-potassium phosphate, pH 7·2; curve *E*, 0·1 M-*N*-ethylmorpholine, pH 7·2; curve *F*, 0·01 M-potassium phosphate, pH 7·2.

plete before combination with formaldehyde could occur. In the presence of 0·01 M-EDTA, or 0·1 mM-8-hydroxyquinoline-5-sulphonate, the rate of tetrahydroPGA decomposition was slowed sufficiently to allow formation of methylenetetrahydropteroylglutamic acid. The subsequent breakdown of this compound was considerably slower than that of tetrahydroPGA under the same conditions (cf. Fig. 7). Spectrum change was also retarded in the presence of higher concentrations of phosphate (0·1 M), probably due also to the binding of heavy metals. In 0·01 N-sodium hydroxide the spectrum change was small (Fig. 13) and a similar result not shown in the figure was obtained in 0·1 M-*N*-ethylmorpholine, pH 9·2. As with free tetrahydroPGA, this is more probably due to the smaller spectrum changes that accompany oxidative degradation at high pH rather than to increased stability at high pH, as was at first thought (Blakley, 1958).

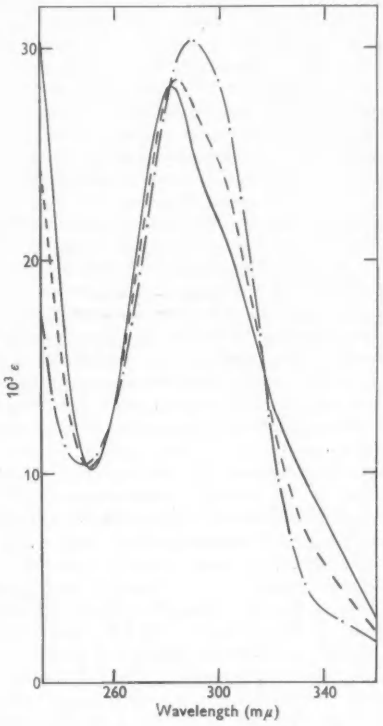


Fig. 14. Absorption spectra of dihydroPGA (0·03 mM) in 0·1 M-potassium phosphate, pH 7·2, containing mM-BAL. ——, No formaldehyde; - - - -, 10 mM-formaldehyde; —·—, 0·12 M-formaldehyde.

Reaction of formaldehyde with dihydropteroylglutamic acid and N^{10}*-formyltetrahydropteroylglutamic acid.* Dihydropteroylglutamic acid, prepared by the method of Futterman (1957), or by catalytic hydrogenation (Blakley, 1957), showed a bathochromic shift and increase in the extinction maximum (Fig. 14) in the presence of formaldehyde with the formation of a product having a dissociation constant of about 0·01 M, which agrees well with that previously obtained by another method (Blakley, 1959). If the product is the N^5-hydroxymethyl derivative, the extinction increase associated with its formation constituted an exception to the general rule of decreased extinction accompanying N^5-hydroxymethyl group formation.

N^{10}-Formyltetrahydropteroylglutamic acid showed only a slight decrease in the extinction maximum (at 260 mμ) at concentrations of formaldehyde from 0·01 to 0·12 M (Fig. 15) but, in addition, there occurred a change in absorption around the inflexion at 300 mμ. The value obtained for the dissociation constant of the product from extinction changes at 310 mμ where N^5-hydroxymethyl formation did not interfere was 16 mM, in good agreement with the previous value (Blakley, 1959).

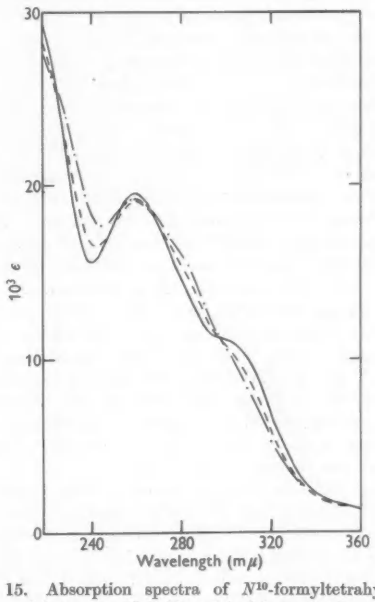


Fig. 15. Absorption spectra of N^{10}-formyltetrahydropteroylglutamic acid (0·03 mM) in 0·1 M-potassium phosphate buffer, pH 7·2, containing mM-BAL. ——, No formaldehyde; - - - -, 10 mM-formaldehyde; —·—, 0·12 M-formaldehyde.

DISCUSSION

In a previous paper (Blakley, 1959) data were reported which indicated that formaldehyde combined with 4-hydroxy-6-methyltetrahydropteridine to form a complex with dissociation constant of 0·03 M. Since N^5-formyl-4-hydroxy-6-methyltetrahydropteridine was found to bind very small amounts of formaldehyde, the dissociation constant of the product being about 0·4 M, the compound formed by 4-hydroxy-6-methyltetrahydropteridine and formaldehyde was considered to be N^5-hydroxymethyl-4-hydroxy-6-methyltetrahydropteridine.

It has now been shown that the spectra of solutions of hydropteridines at pH 7·2 are shifted in the presence of formaldehyde. The evidence indicates the formation of two compounds by the action of formaldehyde on 4-hydroxy-6-methylhydropteridine in the following manner:

$$\text{Hydropteridine} \xrightleftharpoons{+\text{formaldehyde}} \text{complex 1} \xrightleftharpoons{+\text{formaldehyde}} \text{complex 2.}$$

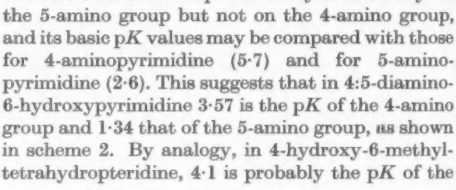
The formation of complex 1, observable in 25 mM-formaldehyde, caused a decrease in absorption as well as a bathochromic shift of the spectrum. Complex 2 was formed at concentrations of formaldehyde above 0·1 M, as indicated by increased absorption in addition to a further bathochromic shift. The dissociation constant of the second product was calculated as approximately 0·2 M.

The high dissociation constant of complex 2 indicated that it was not identical with the complex studied by the chemical method, i.e. N^5-hydroxymethyl-4-hydroxy-6-methyltetrahydropteridine. Complex 1 is therefore presumed identical with the last-named compound. Confirmation that complex 2 is not the N^5-hydroxymethyl derivative was found in the fact that N^5-formyl-4-hydroxy-6-methyltetrahydropteridine also showed increased absorption and a bathochromic shift at high formaldehyde concentrations, and in this case a single product was formed having a dissociation constant of 0·3 M. Chemical analysis previously gave a value of approximately 0·4 M. Evidence has been presented that this product is the N^3-hydroxymethyl derivative, and the similarity of the spectrum changes suggests that complex 2 of 4-hydroxy-6-methyltetrahydropteridine is the N^3N^5-bishydroxymethyl derivative.

It appears from the data on 4-hydroxy-6-methyltetrahydropteridine that formation of the N^5-hydroxymethyl derivative results in decreased absorption. This is also the case when low concentrations of formaldehyde react with N^{10}-formyltetrahydropteroylglutamic acid and with 2-chloro-4-methyl-N^8-benzyltetrahydropteridine. In the last compound only N^5 is available for reaction with formaldehyde, so that the spectrum change when the solution of this compound is treated with formaldehyde must be due to formation of the N^5-hydroxymethyl derivative.

Tetrahydroquinoxaline, like 4-hydroxy-6-methyltetrahydropteridine, showed a two-stage spectrum change in the presence of formaldehyde. This is interpreted as due to formation firstly of N^5-hydroxymethyltetrahydroquinoxaline and secondly, at high formaldehyde concentration, of N^5N^8-bis(hydroxymethyl)tetrahydroquinoxaline. The spectrophotometric data gave a value of 0·08 M for the dissociation constant of the last-named compound. Chemical analysis gave a value of 0·01 M, which presumably applies to the N^5-hydroxymethyltetrahydroquinoxaline.

Although the spectrophotometric results thus suggest formation of the bishydroxymethyl derivative of tetrahydroquinoxaline, no definite evidence has been obtained that formaldehyde reacts with N^8 of hydropteridines. In hydropteridines the N^8-amine group is probably affected by amidine-like resonance which confers much higher basic strength to the N^8-amine group than is possessed by the N^5-amine group, and which might cause a difference in the ability of these groups to react with formaldehyde. The evidence for the difference in basic strength of the N^5- and N^8-amine groups of hydropteridines is as follows. Data obtained for 4-hydroxy-6-methyltetrahydropteridine (scheme 2; III) indicated an acidic pK of 10·2 and one basic pK of 4·1; the other basic pK is lower than 4·1. The structurally related 4:5-diamino-6-hydroxypyrimidine (scheme 2; IV) has an acidic pK of 9·86 and basic pK values of 1·34 and 3·57. To decide which of the basic pK values should be assigned to the 5-amino group, reference is made to the fact that this compound acetylates readily on the 5-amino group but not on the 4-amino group, and its basic pK values may be compared with those for 4-aminopyrimidine (5·7) and for 5-aminopyrimidine (2·6). This suggests that in 4:5-diamino-6-hydroxypyrimidine 3·57 is the pK of the 4-amino group and 1·34 that of the 5-amino group, as shown in scheme 2. By analogy, in 4-hydroxy-6-methyltetrahydropteridine, 4·1 is probably the pK of the



Scheme 2

Table 1. *Summary of approximate dissociation constants for the compounds formed by the reaction of various bases with formaldehyde*

Base	Derivative formed	$10^3 \times$ Dissociation constant (M)	Method employed
Tetrahydroquinoxaline	N^5-Hydroxymethyl	10	Chemical
	N^5N^8-Bishydroxymethyl	80	Spectrophotometric
2-Hydroxypyrimidine	N^3-Hydroxymethyl	400	Spectrophotometric
4-Hydroxy-6-methylpteridine	N^3-Hydroxymethyl	300	Spectrophotometric
4-Hydroxy-6-methyltetrahydropteridine	N^5-Hydroxymethyl	30	Chemical
	N^3N^5-Bishydroxymethyl	200	Spectrophotometric
N^5-Formyl-4-hydroxy-6-methyltetrahydropteridine	N^3-Hydroxymethyl	300	Spectrophotometric
		400	Chemical
2-Chloro-4-methyl-N^8-benzyltetrahydropteridine	N^5-Hydroxymethyl	290	Chemical
		500	Spectrophotometric
Pteroylglutamic acid	N^3-Hydroxymethyl	300	Spectrophotometric
TetrahydroPGA	N^5N^{10}-Methylene	0·03	Spectrophotometric
N^{10}-Formyltetrahydropteroylglutamic acid	N^5-Hydroxymethyl	16	Spectrophotometric
		12	Chemical

N^8-amine group and the pK of the N^5-amine group is probably about 1·4, as represented in scheme 2 (the figures in parentheses referring to pK values for adjacent groupings). This is a more likely interpretation than that previously given (Blakley, 1958).

The dissociation constants obtained for N^5-hydroxymethyl and N^3-hydroxymethyl derivatives of hydropteridines were all relatively high (0·01–0·5M), so that at concentrations of formaldehyde below 10 mM these derivatives would not be formed in significant amounts. Since spectrophotometric studies indicated that 0·05 mM-tetrahydroPGA reacted with formaldehyde at concentrations as low as 0·05 mM and that the reaction was virtually complete in mM-formaldehyde, no interference arose from reaction of formaldehyde at N^3 or N^8 in studies with tetrahydroPGA. The same value of the dissociation constant was obtained at formaldehyde concentrations ranging from 0·05 to 0·15 mM and the spectrum change was reversed by addition of bisulphite to the solution. Together with previous data the spectrophotometric results clearly indicate a reversible reaction which occurs rapidly at neutral pH in the absence of any enzyme.

These results were obtained with mM-BAL used to stabilize the tetrahydroPGA. Since sulphydryl compounds are known to react with formaldehyde, the possibility that the BAL had some influence on the reaction of tetrahydroPGA and formaldehyde must be considered. The presence of BAL did not appear to alter the product formed by reaction of tetrahydroPGA with formaldehyde, since the spectrum obtained in the presence of 10 mM-formaldehyde was the same whether BAL was present or absent. The possibility remains that the value obtained for the dissociation constant of the product is in error, due to reaction of some of the formaldehyde with BAL. When BAL was present at a concentration of 5 mM or higher, high values were certainly obtained for the dissociation constant. Since the value obtained in the presence of 0·5 mM-BAL was the same as in the presence of mM-BAL, interference by BAL at these levels is at any rate slight, if not absent. Assuming that the value of the dissociation constant is not greatly in error, the very low value ($3{\cdot}1 \times 10^{-5}$M) indicates clearly that it is a compound of a type different from the hydroxymethyl derivatives formed by simple hydropteridines; it is presumably N^5N^{10}-methylenetetrahydropteroylglutamic acid. The difference between the value obtained for tetrahydroPGA and those obtained with other bases is readily seen from the summary of data in Table 1.

Since a small amount of tetrahydroPGA is always in equilibrium with methylenetetrahydropteroylglutamic acid, the stability of the latter would be expected to be not much greater than that of tetrahydroPGA, and this was found to be the case. Cation-chelating agents, such as EDTA and 8-hydroxyquinoline-5-sulphonic acid, diminished the rate of breakdown and even phosphate at high concentrations (0·1M) had a similar effect. As with tetrahydroPGA, breakdown occurred at all values of pH, the only difference at high pH being that the spectrum change accompanying breakdown was smaller.

SUMMARY

1. In the presence of increasing concentrations of formaldehyde, the spectrum of 4-hydroxy-6-methyltetrahydropteridine showed a progressive bathochromic shift. At low formaldehyde concentrations (25 mM) the absorption was diminished (believed to correspond to the formation of the N^5-hydroxymethyl derivative), and at higher concentrations of formaldehyde showed an increase.

2. The spectrum shift and increase in the extinction maximum in the presence of high concentrations of formaldehyde was also shown by N^5-formyl-4-hydroxy-6-methyltetrahydropteridine, 4-hydroxy-6-methylpteridine, pteroylglutamic acid and 2-deaminopteroylglutamic acid. The dissociation constants calculated from these spectrum changes ranged from 0·2 to 0·4 M.

3. These data are interpreted as indicating that 4-hydroxypteridines combine with formaldehyde when it is present at a high concentration to form N^3-hydroxymethyl derivatives. This is confirmed by the fact that 2-hydroxypyrimidine behaves similarly but the spectrum of 2-methoxypyrimidine is unaffected by formaldehyde.

4. At neutral pH solutions of tetrahydropteroylglutamic acid rapidly decomposed as shown by spectrum changes. Chelating agents, thiols (especially 2:3-dimercaptopropan-1-ol) and formaldehyde retarded this oxidative decomposition.

5. In the presence of 2:3-dimercaptopropan-1-ol the reaction of tetrahydropteroylglutamic acid with formaldehyde resulted in an increase in the extinction maximum from 28 400 to 32 000 and shift in the wavelength of the absorption maximum from 298 to 294 mμ. The change was reversed by addition of bisulphite.

6. The dissociation constant for the product of this reaction was calculated to be $3{\cdot}1 \pm 0{\cdot}19 \times 10^{-5}$ M.

7. The product of the reaction, believed to be N^5N^{10}-methylenetetrahydropteroylglutamic acid, is also subject to oxidative decomposition at all pH values. Chelating agents retard this decomposition.

8. The changes produced by formaldehyde in the spectra of a number of other hydropteridines are also discussed.

I am greatly indebted to Dr D. J. Brown, who made much of this work possible by his generous gifts of pteridines and pyrimidines. I should also like to express my indebtedness to Dr H. P. Broquist for a gift of leucovorin; to Professor A. H. Ennor and Dr J. F. Morrison for assistance in the preparation of the manuscript; to Professor A. Albert for helpful suggestions concerning the ionisation of hydropteridines and to Mr J. McKeough for skilled technical assistance.

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Nucleoproteins of White Clover

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'Microsomal' ribonucleoproteins have been isolated from several sources, such as liver cells (Petermann & Hamilton, 1957), yeast cells (Chao & Schachman, 1956), pea shoots (Ts'o, Bonner & Vinograd, 1956) and *Escherichia coli* (Tissières & Watson, 1958). Preparations from leaf cells of higher plants have not been reported, although some earlier work has indicated that high-molecular-weight ribonucleoproteins are in fact present. Thus Pirie (1950, 1957) described the preparation from tobacco leaves of a nucleoprotein fraction which was sedimentable by centrifuging at 80 000*g* for 30 min. With clover the bulk of the ribonucleic acid of leaf extract could be sedimented at 100 000*g* in 40 min., and, when redissolved, this sediment was shown in the analytical ultracentrifuge to contain a component of sedimentation coefficient 65 s (Lyttleton, 1956).

The studies which have been made on the 'microsomal' ribonucleoprotein particles from the various sources mentioned above have indicated that these particles are stable in solution only within a limited range of conditions, and it is probable that the lack of success in earlier attempts to isolate high-molecular-weight ribonucleoprotein from clover (Lyttleton, 1956) was due to a failure to recognize this difficulty.

With the knowledge now available of the conditions necessary to prevent degradation of the particles, their isolation has been re-investigated. This paper describes the successful isolation of 'microsomal' ribonucleoproteins from clover leaf. It gives details of their physical and chemical properties, which are shown in many respects to be similar to those of ribonucleoproteins isolated from other sources.

MATERIALS AND METHODS

Plant material. White clover (*Trifolium repens* L.) was grown in boxes in glasshouses. Three or four crops of mature leaves were taken before the plants were discarded for new

sowings. Field-grown clover was unsatisfactory because of the prevalence of infection with pea-wilt virus (Fry, 1959), which contaminated any preparation of ribonucleoprotein.

Extraction of leaves. The leaves were usually ground in an End Runner Mill as previously described (Lyttleton, 1956). Alternatively, the leaves, moistened with a suitable buffer solution, were pressed through an annular aperture (0·001 in.) in a chilled stainless-steel press (Pirie, 1956). The paste of disintegrated tissue thus produced was filtered through Whatman no. 3 MM paper in a basket centrifuge and the residue washed with buffer solution. This method was particularly useful when small amounts of concentrated extract were required, but the chloroplast material was so highly dispersed that its removal by fractional centrifuging was never as complete as from solutions produced by grinding.

Fractional centrifuging. This was carried out in a Spinco model L preparative ultracentrifuge (Beckman Instruments, Spinco Division, Palo Alto, Calif., U.S.A.). Figures quoted for the centrifugal force are those tabulated for the average radius of rotation.

All manipulations were carried out at 4°, and a precooled rotor was used in the centrifuge, to which refrigeration was applied during sedimentation. Resuspension of the pellets was effected with the aid of a magnetic stirrer, and could be made more rapid by filling and emptying a 10 ml. hypodermic syringe with the needle inserted below the level of the resuspending medium. The rapid flow of liquid through the rather narrow orifice was most effective in dispersing the gel-like sediment produced by high-speed centrifuging. A hypodermic syringe was also used to withdraw the supernatants from the clarifying stages, as the sediments produced here were not well packed.

Ultracentrifugal analyses. These were carried out in an air-driven ultracentrifuge constructed for this Division by the Dominion Physical Laboratory. It was fitted with an inclined-bar Philpot schlieren optical system, and operated under automatic-speed control at 42 000 or 60 000 rev./min. with a constancy of 1 part in 10 000. Sedimentation coefficients are expressed in Svedberg units (s).

Electrophoretic analyses. These were carried out at 0·5° in a Tiselius apparatus manufactured by LKB-Produkter Ltd. Mobilities are expressed in units of 10^{-5} cm.^{2}/v sec.

Absorption spectra. These were measured on a Beckman DU quartz spectrophotometer. In describing the ultraviolet spectra of the nucleoprotein preparations, the ratio $E_{max.} : E_{min.}$ expresses the ratio of the extinctions at the maximum (near 260 mμ) and at the minimum (usually 230–240 mμ).

Ribonucleic acid. The ribonucleic acid (RNA) content of samples was estimated by precipitation with 5% (w/v) trichloroacetic acid, washing with 70% (v/v) ethanol and hydrolysing with 2N-KOH at 20° for 18 hr. The resulting nucleotides were separated by paper chromatography in propan-2-ol–aq. NH_3 soln. (Markham & Smith, 1952), and their concentration was determined after elution by means of their ultraviolet absorption.

Base analysis of ribonucleic acid. This was carried out by hydrolysis in 1N-HCl for 1 hr. at 100° followed by paper chromatography in propan-2-ol–HCl (Smith & Markham, 1950).

Phosphorus. This was estimated by the method of Allen (1940).

Buffer solutions. Phosphate buffers contained analytical-grade Na_2HPO_4 and KH_2PO_4, and their concentration is expressed in terms of ionic strength (*I*). Reagent-grade 2-amino-2-hydroxymethylpropane-1:3-diol (tris) was used for making tris–HCl buffers. Other salts used were of analytical grade.

Direct examination of extracts

An extract from clover was prepared from 30 g. of leaf moistened with 5 ml. of water in the stainless-steel press. Examination of this extract, diluted with an equal volume of water, in the analytical ultracentrifuge revealed two heavy components (Fig. 1*a*) with apparent sedimentation coefficients 65s and 55s, as well as large concentrations of the two lower-molecular-weight components, fractions I and II (Singer, Eggman, Campbell & Wildman, 1952). Experiments which will be described later indicated that at least one, and probably both, of these two heavy components were ribonucleoproteins. Accordingly they will be referred to in the remainder of this paper as NP1 and NP2 respectively.

When the extract was diluted with phosphate buffer, pH 7·5, *I* 0·2, instead of with water, NP2 was no longer detectable and NP1 was somewhat reduced in concentration. After dialysis of the extract against phosphate buffer, pH 7·5, *I* 0·1, neither heavy component was detectable in the ultracentrifuge pattern.

Isolation of NP1 and NP2

Precipitation. Previous work (Lyttleton, 1956) had shown that leaf nucleoprotein could not be recovered after precipitation by addition of Na_2SO_4 or by lowering of the pH of the solution. Precipitation by addition of ethanol at −5° to a concentration of 20% (v/v) permitted recovery of the nucleoprotein. Attempts were therefore made to concentrate selectively NP1 and NP2 by ethanol precipitation at concentrations between 15 and 25%, but although good recovery of NP1 was obtained, the ratio of NP1 to fraction I was not increased. The second component, NP2, could not be recovered after ethanol precipitation.

Fractional centrifuging. Because of their high sedimentation rates, NP1 and NP2 may be selectively concentrated by centrifuging at high speeds. Ultracentrifuge examination showed that sedimentation at 105 000*g* for 60 min. removed all but a trace of these components from the supernatant solution. Accordingly the scheme outlined in Fig. 2 was used for isolating NP1 and NP2 from clover extracts. The terminology of Ts'o *et al.* (1956) is used, where DC1, DC2 and DC3 represent preparations obtained by differential centrifuging to one, two and three stages.

The choice of medium for extraction and resuspension was most important, an ideal medium being one which would produce good yields of intact NP1 and NP2, and prevent the production of artifacts. It was found that values between pH 6·0 and 7·5 were most satisfactory, as below pH 6·0 the components tended to aggregate, and above pH 7·5 they tended to break down to lower-molecular-weight material.

Even within this favourable range of pH values, NP1 and NP2 were unstable in the presence of buffers of ionic strength greater than 0·02. This instability was greatly reduced by the addition of low concentration of Mg^{2+} ions to the solution, and most preparations were made in the presence of mM-$MgSO_4$.

The media which were used during these investigations may be divided into three classes.

Low-ionic-strength medium. An extract prepared by grinding clover into water had a pH close to 6·0 when freshly prepared, though on standing it dropped rapidly. It was thus possible to fractionate the components of a clover extract with only water as extraction and resuspension medium. However, in DC2 and DC3 preparations made in this way, in addition to NP1 and NP2 the solution contained a new component of sedimentation coefficient 40–42s (Fig. 1*b*). This component, not detectable in the original extract, was considered to be an artifact. The use of mM-$MgSO_4$ in place of water gave similar results, with a slightly reduced production of the 40s component.

High-ionic-strength medium. Provided that magnesium was present, it was found possible to use buffered 0·1M-NaCl to prepare NP1, though NP2 did not survive sedimentation in this medium. No new components were

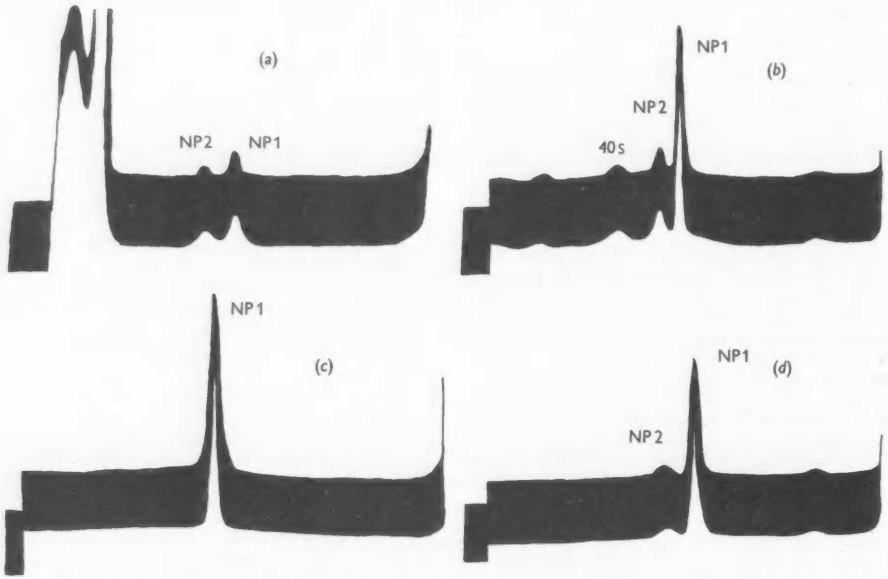

Fig. 1. Ultracentrifuge diagrams. (*a*) Clover-leaf extract, diluted with an equal volume of water. (*b*) DC2 fraction prepared in water. (*c*) DC3 fraction prepared in 0·1M-NaCl–mM-$MgSO_4$–phosphate buffer (pH 7·5, *I* 0·02). (*d*) DC3 fraction prepared in mM-$MgSO_4$–phosphate buffer (pH 7·5, *I* 0·02). All diagrams were photographed 9 min. after running speed (42 000 rev./min.) was reached. Sedimentation is from left to right.

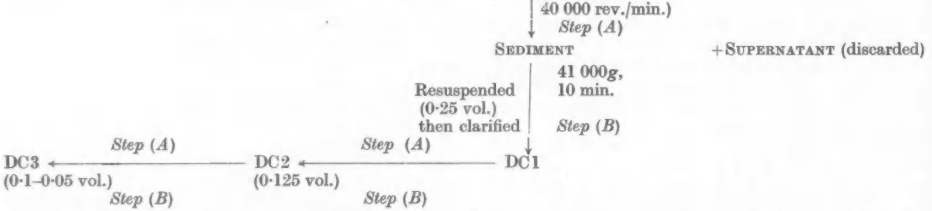

Fig. 2. Fractionation of clover extract by differential centrifuging. In some preparations, where a larger volume was to be handled, the clarification of the original extract and the first sedimentation were carried out in the no. 30 rotor for 20 min. at 20 000 rev./min. (35 000 *g*) and 120 min. at 30 000 rev./min. (78 000 *g*) respectively.

produced, and the best preparations of NP1 have been made in 0·1 M-NaCl–mM-$MgSO_4$ buffered at pH 7·5 with phosphate (*I* 0·01). A DC3 preparation made with this medium was seen to contain only NP1 when examined in the analytical ultracentrifuge (Fig. 1*c*).

Medium-ionic-strength medium. In order to preserve NP2 and yet avoid the production of artifacts some compromise between low- and high-ionic-strength media seemed desirable. A range of these was tried, mainly with total ionic strength 0·02, made up of phosphate buffer, pH 6·5, 7·0 or 7·5, or tris buffer at pH 7·2, either with or without 0·01 M-NaCl but always containing mM-$MgSO_4$. With these media the production of artifacts was avoided, but with none of them was a satisfactory recovery of NP2 found (Fig. 1*d*). Each stage of centrifuging led to a decrease in the ratio of concentrations NP2 : NP1, so that whereas in fresh extract this ratio was approx. 1 : 2, in DC3 preparations it dropped to approx. 1 : 10.

In all media of low or medium ionic strength which contained mM-$MgSO_4$, DC2 and DC3 preparations contained small amounts of a component of sedimentation coefficient 100–105 s. A similar component has also been produced by fractional centrifuging of pea-shoot extracts (Ts'o *et al.* 1956) and of bacterial extracts (Tissières & Watson, 1958), and is apparently a dimer of NP1.

The method of preparing NP1 and NP2 depended upon the object of the experiment. For studies on the properties of NP1, sedimentation was carried out in buffered 0·1 M-NaCl–mM-$MgSO_4$. Three stages of sedimentation were needed to remove fraction I completely from the final preparation. However, the two-stage preparation (DC2), containing about 10% of fraction I, was suitable for preliminary work on the dissociation of NP1. On the other hand, solutions of medium ionic strength, as mentioned above, were used in making preparations to study the properties of NP2, which was always accompanied by larger amounts of NP1.

It was difficult to remove the last traces of highly dispersed chloroplastic material even from DC3 preparations. If a high yield was sought by prolonged grinding of leaf tissue, or by passing larger amounts of leaf through the stainless-steel press, this was invariably accompanied by the production of chlorophyll-containing fragments with sedimentation rate only slightly greater than those of the desired components. Under these circumstances, even the four stages of clarification used in producing the DC3 preparation did not completely remove contaminating material. It should be noted, however, that this contamination did not hinder observation either in the analytical ultracentrifuge or in the electrophoresis apparatus.

Distribution of ribonucleic acid during fractionation

With the chromatographic isolation technique described above, estimations were regularly made of the RNA content of the clarified extract and of the final product, DC2 or DC3. In most cases between 25 and 35% of the RNA contained in the clarified extract was recovered in a DC3 preparation, and slightly more (30–40%) in a DC2 preparation. Usually the higher recoveries were associated with the use of medium ionic-strength extraction media; but the results were somewhat irregular, and differences were probably due to minor variations in manipulative techniques.

On several occasions a more complete balance sheet was made of the distribution of RNA during a fractionation, and a typical result is given in Fig. 3.

Ultraviolet spectrum

Whereas the ultraviolet spectrum of clarified clover extract showed a continually increasing absorption with decreasing wavelength, with a shoulder in the region 270–280 mμ, that of a DC2 or DC3 preparation showed a pronounced maximum at 260 mμ, indicating that one or both of the heavy components which were concentrated in these fractions contained nucleic acid, and were presumably nucleoprotein in nature.

The actual shape of the ultraviolet-absorption spectrum depended on the degree of contamination with non-nucleoprotein material, such as fraction I (in DC2 preparation) and residual chloroplast fragments. The best DC3 preparations gave a ratio $E_{max.}: E_{min.}$ of 1·5–1·55 : 1, with a minimum absorption at 233 mμ.

RESULTS

Properties of NP1 and NP2

Stability. All preparations have proved to be somewhat unstable even at 4°. This instability is revealed by ultracentrifugal examination, which shows a progressive loss of NP1 and NP2 with the production of degraded material of ill-defined sedimentation behaviour, some aggregated and sedimenting at low rotor speeds and some of low molecular weight not separating from the meniscus of the ultracentrifuge cell at 60 000 rev./min. The NP2 component is particularly labile, and rarely persists as long as 18 hr. after completion of a fractionation. The NP1 component, however,

Clarified extract (25·2 mg.)
↓
DC1 (13·5 mg.) + residue (5·10 mg.) + supernatant 1 (4·78 mg.)
↓
DC2 (8·9 mg.) + residue (2·5 mg.) + supernatant 2 (1·02 mg.)
↓
DC3 (7·6 mg.) + residue (0·25 mg.) + supernatant 3 (0·80 mg.)

Fig. 3. Distribution of RNA as determined by nucleotide chromatography during differential centrifuging of clover extract. This fractionation was carried out with phosphate buffer (pH 7·0, *I* 0·01)–0·01 M-NaCl–mM-$MgSO_4$ throughout. The 'residues' referred to are the discarded sediments produced during the clarifying steps in the fractionation.

usually remains substantially unaltered for 24 hr. and may persist for 2 or 3 days at 4°.

Pirie (1950) found that nucleoprotein derived from tobacco leaf liberated acid-soluble phosphate on standing. Similarly the degradation of NP 1 and NP 2 is accompanied by a decrease in the amount of RNA and of phosphate which can be precipitated from the solution by cold 5 % (w/v) trichloroacetic acid. In a typical case, from a freshly prepared DC 3 preparation in tris-HCl buffer, 95 % of the RNA and 97 % of phosphate could be precipitated; these amounts decreased progressively until after 3 days at 4°, 71 % of the RNA and 69 % of phosphate were precipitable.

Apart from the requirement for magnesium to prevent dissociation in the presence of buffer salts, no definite relationship has been established between suspension medium and stability of NP 1 and NP 2, and no satisfactory way of decreasing breakdown of the components has been found. As a result of this, all investigations of the intact components have been carried out within 24 hr., and when possible within 2–3 hr. of their isolation.

Sedimentation. The sedimentation coefficient of NP 1, determined in 0·1 M-NaCl–mM-$MgSO_4$ buffered with phosphate at pH 7·5, I 0·01, showed a moderate dependence on concentration, rising from 76 s at a concentration of 8 mg./ml. to an extrapolated value of 83 s at infinite dilution. The lower value of 65 s observed for NP 1 in clover extracts is due to the fact that no correction was made for the high concentration of protein in which sedimentation was proceeding.

Measurement of the sedimentation coefficient of NP 2 in DC 3 preparations gave values of 62–66 s. If the effect of concentration upon sedimentation is the same as with NP 1, the value at infinite dilution might be expected to be between 68 and 70 s.

Electrophoresis. Electrophoretic analyses of NP 1 have normally been carried out in the buffer solution which was used for differential centrifuging, thus eliminating the need for dialysis. In phosphate buffers, pH 6·0–7·5, I 0·01, containing 0·01 M-NaCl and mM-$MgSO_4$, NP 1 migrated as a single symmetrical boundary with a mobility of $-9{\cdot}8$ units at pH 7·5 and $-8{\cdot}1$ units at pH 6·0. Behind the main NP 1 component moved a small boundary, accounting for 10–15 % of the total area, apparently produced by contaminating dispersed chloroplastic material. The chlorophyll remaining in the solution migrated with this boundary (Fig. 4).

It is difficult to study the electrophoretic behaviour of NP 2 because of its lability. Various preparations made with the object of discovering whether or not it migrated independently of NP 1 led to inconclusive results, because ultracentrifugal examination of the residues after electrophoretic analysis showed that at most a trace of NP 2 remained.

Such evidence as is available indicates that in fact NP 1 and NP 2 migrate together on electrophoresis. On no occasion were the boundaries produced from DC 2 or DC 3 preparations containing small residual amounts of NP 2 observed to split, indicating the presence of more than one component. In one analysis of a DC 3 preparation containing an appreciable amount of NP 2, material migrating with the NP 1 electrophoretic boundary was isolated from the ascending limb of the U-tube, and in the ultracentrifuge it was shown to contain a small amount of NP 2 as well as a larger amount of NP 1.

In an attempt to verify this finding, the product of the first step in the centrifugal fractionation (DC 1), which contained a higher proportion of NP 2 than later steps, was electrophoretically analysed immediately after its preparation. The electrophoretic pattern showed a boundary with a mobility approximately that of NP 1, which split into two as analysis proceeded. The components forming this double boundary were isolated from the ascending limb of the U-tube, and examined in

Fig. 4. Electrophoretic analysis of NP 1, in 0·01 M-NaCl–mM-$MgSO_4$–phosphate buffer (pH 7·0, I 0·01). (*a*) Anode limb; (*b*) cathode limb.

the ultracentrifuge. Along with the expected NP1, this showed a new 40s component, possibly similar to the artifact produced by using water as medium during fractionation, but no NP2 remained. The ultraviolet spectrum showed a ratio $E_{max.}$: $E_{min.}$ of 1·28 : 1, much lower than that of good NP1 preparations, so the 40s artifact presumably contained some of the fraction I protein with which DC1 preparations are heavily contaminated.

Content of ribonucleic acid. On three occasions, preparations of NP1 were run in the electrophoresis apparatus, short intermediate cells being used to form the U-tube so that, at the conclusion of the run, electrophoretically pure samples of NP1 could be isolated in the upper section of the ascending limb.

Because the amount available was so small, these colourless pure samples were analysed for RNA by a non-destructive method, similar to that of Englander & Epstein (1957), by determining the extinction at 260 mμ, and measuring the concentration of NP1 by freeze-drying and weighing. The results obtained for the three preparations were 53, 56 and 52 mg. of RNA/100 mg. of NP1. By contrast, DC3 preparations of NP1, which were shown by electrophoresis to contain 10–15% of contaminant, contained 40–45% of RNA.

The ultraviolet-absorption curve of the electrophoretically purified samples of RNA showed the expected maximum at 260 mμ, and a ratio $E_{max.}$: $E_{min.}$ of 1·65–1·70 : 1 (cf. unpurified preparation 1·5–1·55 : 1).

Composition of ribonucleic acid base. The freeze-dried samples of electrophoretically purified NP1 were pooled and hydrolysed with N-HCl at 100° for 1 hr. Chromatography in propan-2-ol–HCl enabled the ratio of the base components of the RNA to be determined, with results given in Table 1. For comparison, the ratios determined on a hydrolysed sample of a DC3 preparation of NP1, before electrophoretic purification, are also given. It has not yet been possible to isolate NP2, so its composition is unknown.

Dissociation products of NP1

As with the ribonucleoprotein particles prepared from yeast (Chao, 1957), from pea shoot (Ts'o, Bonner & Vinograd, 1958) or from *E. coli* (Tissières & Watson, 1958), the leaf ribonucleoprotein NP1 will dissociate in certain solvents into smaller components. The various types of dissociation which have been studied in the analytical ultracentrifuge are considered here according to the solvent which produced them.

Phosphate buffers in the presence of magnesium. Provided that mM-$MgSO_4$ was present, NP1 was not affected by phosphate, pH 7·5, at I below 0·03. When I was 0·05, a small amount of material of low molecular weight was formed, and at I 0·10 two new components were formed, with apparent sedimentation coefficients of 34s and 52s, as well as more low-molecular-weight material. At ionic strengths above 0·5, NP1 was completely dissociated to give a little 34s component, a larger amount of low-molecular-weight material and aggregated material which sedimented at low rates of centrifuging.

The distribution of the RNA in the completely dissociated NP1 was investigated by measuring the ultraviolet-absorption spectrum of the supernatants and redissolved sediments from the analytical ultracentrifuge cell at the conclusion of runs of sufficient length to sediment the 34s component fully. The results, given in Table 2, indicate that the bulk of the RNA after dissociation was found in the unsedimented low-molecular-weight material. The ratio $E_{max.}$: $E_{min.}$ for this latter was 1·82 : 1, indicating that less protein was present per unit of RNA than in the undissociated NP1.

Phosphate buffers in the absence of magnesium. If a solution of NP1 was prepared in the absence of magnesium by carrying out the final steps of the differential centrifuging in water, the addition of phosphate buffer, pH 7·5, I 0·025, produced a partial dissociation of NP1 into two well-defined smaller components (Fig. 5*a*). Their sedimentation rate indicated that they were the same as the 34s and 52s components described above; but in the absence of added magnesium they were produced at lower concentrations of phosphate, appeared less diffuse in the ultracentrifuge and were not accompanied by the formation of material of low molecular weight. The formation of these components was apparently very rapid, for whereas the presence of mM-$MgSO_4$ in solution prevented the dissociation of NP1 by phosphate, pH 7·5, I 0·025, the addition

Table 1. *Base composition of ribonucleic acid of NP1*

	Ratio of base components			
	Guanine	Adenine	Cytosine	Uracil
Electrophoretically pure NP1	1·32	0·95	0·84	0·88
DC3 preparation of NP1	1·26	0·94	0·88	0·92

Table 2. *Distribution of ribonucleic acid after centrifuging of NP1 in analytical cell before and after dissociation by phosphate*

Ionic strength of phosphate (pH 7·5)	E_{260} (supernatant)	E_{260} (sediment)
0·01	0·6	7·5
0·5	5·5	2·2

of $MgSO_4$ to this concentration immediately after the addition of phosphate did not appreciably reverse the dissociation.

Increase of phosphate concentration to I 0·05 did not give greater concentration of the 34s and 52s components; but they became less well defined in the ultracentrifuge, and a little material of low molecular weight was formed. This effect was also brought about by adding 0·1M-NaCl to the phosphate at I 0·025.

Higher concentrations of phosphate lead to progressive loss of NP1 and the production of low-molecular-weight material in a manner very similar to that observed in the presence of magnesium. The bulk of the RNA was again associated with the low-molecular-weight material.

Dialysis of a solution of NP1 against phosphate buffer, pH 7·5, I 0·025, for 18 hr. led to dissociation with the production of a higher proportion of the 34s component than was formed by simple addition of phosphate. Electrophoretic analysis of this dialysed solution revealed two well-defined boundaries, migrating with mobilities $-12{\cdot}5$ and $-9{\cdot}8$ units. The lower mobility agreed with that of undissociated NP1 in a buffer of the same pH and ionic strength which contained mM-$MgSO_4$, so it appeared that the dissociation products moved in the more rapid boundary. It will be noted that no boundary was observed to move with a mobility typical of free RNA in this medium (-14 to -15 units) so the dissociation products were presumably still nucleoproteins. This observation agrees with the findings of Ts'o *et al.* (1958), whose ultracentrifuge studies with an ultraviolet-absorption optical system indicated that similar dissociation products of pea-shoot ribonucleoproteins were themselves nucleoprotein in nature.

Dissociation in tris–hydrochloric acid buffers. A solution of NP1 was prepared in tris–HCl buffer, pH 7·2, I 0·02, containing mM-$MgSO_4$, then dialysed for 18 hr. against the same buffer without $MgSO_4$. The NP1 component was completely dissociated into a component of sedimentation coefficient approximately 50s, together with low-molecular-weight material (Fig. 5*b*). Electrophoretic analysis of this dialysed preparation in tris–HCl (pH 7·2, I 0·03)–0·02M-NaCl revealed components with mobilities $-9{\cdot}0$ and $-14{\cdot}0$ units. The more rapidly moving component was isolated at the end of the run, and was shown to contain the low-molecular-weight material observed in the centrifuge. Its absorption spectrum showed a ratio $E_{max.} : E_{min.}$ of 2·35 : 1. This was considerably higher than the ratio obtained for the low-molecular-weight material produced by dissociation with phosphate, indicating a higher proportion of RNA to protein.

With bacterial ribonucleoprotein particles it has been found that whereas dissociation occurs in the absence of magnesium, in tris–HCl buffers containing 5 mM-$MgSO_4$ the particles aggregated to produce a heavier component, presumably a dimer, of sedimentation coefficient approximately 110s (Tissières & Watson, 1958). A similar aggregation was not produced when preparations of NP1 were dialysed against tris–HCl buffer (pH 7·2, I 0·03)–5 mM-$MgSO_4$. The small amount of 105s component normally present in DC3 preparations was not increased by the higher concentration of magnesium.

Action of chelating compounds. Removal of magnesium from a solution of NP1 in phosphate buffer (pH 7·5, I 0·01)–mM-$MgSO_4$ by addition of a slight excess of sodium ethylenediaminetetra-acetate brought about considerable loss of NP1, apparently by aggregation, but produced no recognisable products of dissociation. A similar effect was obtained with sodium citrate.

DISCUSSION

The experiments described in this paper indicate that clover-leaf cells contain a class of high-molecular-weight ribonucleoprotein particles (NP1) which have many properties in common with similar particles found in other cell extracts. The

Fig. 5. Ultracentrifuge diagrams. (*a*) NP1 in phosphate buffer, pH 7·5, I 0·025; (*b*) NP1 dialysed against tris–HCl buffer, pH 7·2, I 0·02. Both diagrams were photographed 9 min. after running speed (42 000 rev./min.) was reached. Sedimentation is from left to right.

RNA content of NP1 (52–56%) is intermediate between the values found for particles from *E. coli* (60–65%; Tissières & Watson, 1958) and those from yeast (40–45%; Chao & Schachman, 1956) and from pea shoots (35–40%; Ts'o *et al.* 1956). The extrapolated sedimentation coefficient of NP1 (83s) also agrees with those found for ribonucleoproteins from the sources mentioned. A further similarity lies in the stabilizing effect of Mg^{2+} ions on NP1, and in its dissociation into large subunits in the presence of phosphate buffers at pH 7·5.

The NP1 particles from clover leaf appear to be considerably less stable than those described from other sources. As it is known that ribonucleoproteins of this type are susceptible to the action of ribonuclease (Pirie, 1950; Ts'o *et al.* 1956), it is possible that the instability observed here is due to contamination with ribonuclease, which is known to occur in leaves of higher plants (Holden & Pirie, 1955). This would explain the production of acid-soluble RNA and phosphorus from the NP1 preparations on standing.

Earlier work on leaf proteins (Eggman, Singer & Wildman, 1953) had indicated that when fraction I was isolated by fractional centrifuging, the bulk of the soluble RNA was found to be associated with this component rather than with heavier molecules of the type found here. This may be accounted for by the fact that the preparations were sedimented and resuspended in buffer at pH 7·0 in the absence of magnesium, and were dialysed against similar buffers before ultracentrifugal analyses. These treatments might be expected to degrade the heavy ribonucleoproteins and to lead to contamination of fraction I. As shown previously (Lyttleton, 1956; Lyttleton & Ts'o, 1958), it is possible by mild methods to prepare fraction I free from RNA.

It is doubtful whether all of the soluble RNA of leaf cells is located in NP1 and NP2. The distribution observed during fractionation indicated that some 20% of the RNA in the clarified extract was not removed from the supernatant under conditions for which sedimentation of NP1 and NP2 was substantially complete. The rapidity with which the fractionation was carried out makes it unlikely that the unsedimented RNA was merely a product of attack by ribonuclease on the NP1 and NP2.

For the study of ribonucleoprotein particles of plant origin, leaf tissue has several disadvantages as a starting material. In addition to the instability discussed above, the purification of the ribonucleoproteins by differential centrifuging is made more difficult by the presence of large amounts of relatively high-molecular-weight protein (fraction I, sedimentation coefficient 17–18s) and of finely dispersed chloroplast fragments. Three stages of sedimentation are needed to remove fraction I, and even then contamination with chloroplast fragments is often troublesome.

Extracts of leaf cells contain two soluble high-molecular-weight components, in place of the more usual single component observed in etiolated shoots and in roots. The presence of this second component is not confined to clover leaves; examination of extracts from leaves of green peas, Chinese cabbage, spinach, vegetable marrow and Italian ryegrass (Lyttleton, unpublished work) has revealed the presence of a pair of high-molecular-weight components similar to the NP1 and NP2 of clover leaf.

The nature of the second component, NP2, has not been established. During this work it has been considered probable that it is a ribonucleoprotein, but confirmation of this must await its isolation and analysis. So far the only clues as to its nature have been (*a*) its high sedimentation coefficient, which is similar to those for naturally occurring ribonucleoproteins, (*b*) some indication that its electrophoretic mobility is the same as that of NP1, and higher than that of other leaf proteins, and (*c*) its great instability in the presence of buffer salts. This instability explains the fact that it was not observed during previous work with clover-leaf extract, where extraction into buffers was the standard procedure (Lyttleton, 1956).

It is not likely that NP2 is a product of dissociation of NP1. It is observed immediately after extraction in leaf extracts, in which its concentration decreases rather than increases on standing. Its sedimentation coefficient and electrophoretic mobility do not agree with those of the larger fragments of NP1 obtained by dissociation in the presence of phosphate buffers. It appears to be a genuine constituent of leaf cells, though a very unstable one, and its isolation will present a difficult problem.

As has been pointed out by Pirie (1950), the presence in leaf cells of high-molecular-weight ribonucleoproteins of this type should be borne in mind when plant viruses are to be prepared from leaf extracts. Unless some steps are taken to remove these normal leaf constituents they are liable to contaminate any virus prepared by fractional centrifuging alone. The experiments described in this paper, along with other similar unpublished results obtained with other plants, indicate that solution in phosphate buffers of ionic strength greater than 0·2 at pH 7·5 should lead to complete degradation of the ribonucleoproteins from the leaves of most plants, whereas many viruses remain unaffected by this treatment. Alternatively, it should be possible to make use of chelating agents such as ethylenediaminetetra-acetic acid to remove Mg^{2+} ions from solution, hence rendering the ribonucleoproteins unstable.

SUMMARY

1. Ultracentrifugal examination of clover-leaf extracts has shown two components of high sedimentation coefficient to be present.

2. The heavier of these has been isolated and shown to be a ribonucleoprotein containing 50–55% of ribonucleic acid. Its properties have been studied by ultracentrifuging and electrophoresis.

3. In common with similar ribonucleoproteins from pea shoots, yeast or liver cells, this component dissociates into subunits in the presence of certain buffer solutions.

4. The second component is considerably less stable, and has not yet been isolated. It is suggested that it may also be a ribonucleoprotein.

5. The possibility of contamination of plant-virus preparations with these normal constituents of leaf is discussed.

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The Estimation of Aminoacetone and δ-Aminolaevulic Acid

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Since the discovery of δ-aminolaevulic acid as a precursor of porphyrins (Shemin & Russel, 1953; Neuberger & Scott, 1953) two methods for the determination of this compound have been published. One of these involves the conversion of the amino ketone into a pyrrole by condensation with a second ketone such as acetylacetone or ethyl acetoacetate; the pyrrole so formed is then estimated by means of the Ehrlich reaction. Mauzerall & Granick (1956) used a modified Ehrlich reagent, which gave more stable colours and greater sensitivity than that described by Shuster (1956), and their procedure is generally the method of choice, particularly when small amounts of amino ketone are to be measured. It suffers, however, from the disadvantage that any excess of Ehrlich-reacting compounds such as porphobilinogen or indole derivatives must first be removed.

The other method for the determination of δ-aminolaevulic acid is based on the observation (Shuster, 1956) that when the amino ketone is mixed with a solution of picric acid and excess of sodium hydroxide is added, a complex is formed which gives a red–brown colour on subsequent acidification with hydrochloric acid. The mechanism of the reaction and the nature of the complex are not known.

During work on the production of aminoacetone by *Staphylococcus aureus* (Elliott, 1958, 1959), it was necessary to have a suitable assay method. Aminoacetone could be estimated by both procedures, but the picrate method was chosen because of its greater freedom from interference from other substances and because of its simplicity. However, the original procedure (Shuster, 1956) gave erratic results in the determination of both δ-aminolaevulic acid and aminoacetone and the conditions of the estimation were therefore re-investigated.

The present paper describes a simple procedure for the determination of both of these metabolites; although it is less sensitive than the procedure of Mauzerall & Granick (1956), the method is valuable since it can be used in conditions where the latter is inapplicable. It has the additional advantage that, when combined with a simple resin treatment, only amino ketones, among biologically occurring

compounds, are known to react. Methods for the separation of δ-aminolaevulic acid and aminoacetone are also described.

MATERIALS

δ-Aminolaevulic acid hydrochloride was a Parke Davis and Co. product kindly given by Dr J. E. Falk of C.S.I.R.O., Canberra. Aminoacetone hydrochloride was synthesized by the method of Gabriel & Colman (1902). Aminoacetone toluene-*p*-sulphonate was prepared by adding excess of toluene-*p*-sulphonic acid in ethanol to an ethanolic solution of aminoacetone hydrochloride. The salt was crystallized by the cautious addition of ether and was twice recrystallized from the same solvents. M.p. 130·5° (uncorr.) (Found: C, 49·1; H, 6·4; N, 5·9; S, 12·9%. $C_3H_7ON,C_7H_8O_3S$ requires: C, 49·0; H, 6·2; N, 5·7; S, 13·1%).

RESULTS

Absorption spectra of coloured complexes. In the original procedure with picric acid it was recommended (Shuster, 1956) that measurements of the extinction of the coloured complex formed by δ-aminolaevulic acid (ALA) be made at 450–470 mμ. However, the absorption due to picric acid at these wavelengths (Fig. 1) is such that a slight error in the amount of picric acid added can introduce a large error in the estimation and markedly affect the reproducibility. These difficulties may be avoided if measurements are made at 495 mμ, where the absorption due to picric acid becomes negligible; although there is a slight loss in sensitivity (cf. spectrum of amino ketone–picric acid complex, Fig. 1), this modification avoids the necessity of removing the excess of picric acid as recommended by Laver, Neuberger & Udenfriend (1958).

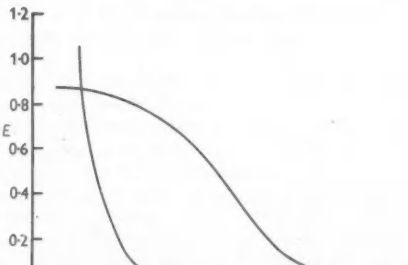

Fig. 1. Absorption spectra of amino ketone picrate complexes. Upper curve: absorption spectrum given by 0·5 μmole of ALA in the standard procedure described in the text measured against the picrate-reagent blank. Aminoacetone gives a curve identical in shape but a few per cent lower in absorption values, and is not shown. Lower curve: spectrum of the picric acid blank measured against water.

Effect of reaction time. The length of time interval between the addition of NaOH and the subsequent acidification with HCl was found to influence greatly the colour yields obtained from ALA and aminoacetone. Under the conditions of the original method the colour yield from ALA is maximal if the HCl is added approx. 45 sec. after the NaOH (Fig. 2, curve 1). Increase of this time causes a marked decrease in colour yield on subsequent acidification; thus after 4 min. the intensity of colour falls to half that obtained after a 45 sec. interval.

The production of colour from aminoacetone under the same conditions is even more sensitive to the reaction time than it is with ALA. Maximum values (Fig. 3, uppermost curve) are obtained after 30 sec. in alkali, the colour decreasing to 50% after 90 sec. This rapid fall in colour yield probably accounts for the report that aminoacetone is inactive in the picrate reaction (Shuster, 1956).

Effect of concentration of sodium hydroxide. The formation of the colour-yielding complex from ALA proceeds more slowly at lower concentrations of NaOH. The final level attained, however, is the same at concentrations of NaOH from 1·5 to 0·75 N, while at 0·38 N this value falls by only 20%. It is also clear from Fig. 2 that the colour-yielding complex is more stable at lower NaOH levels, so

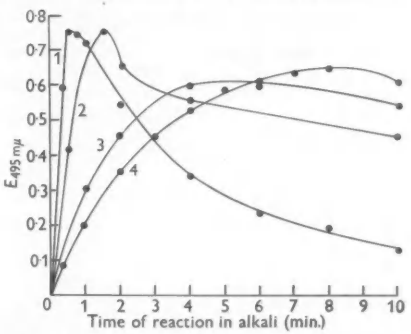

Fig. 2. Effect of reaction time in alkali on colour yield from ALA at different concentrations of NaOH. Colours were developed at room temperature (22°) as follows: 0·5 μmole of ALA hydrochloride in a 2·5 ml. sample was treated with 0·25 ml. of saturated picric acid solution followed by 0·5 ml. of NaOH of appropriate normality (see below). 1·75 ml. of HCl was added at varying times and colours read at 495 mμ against a reagent blank. The HCl solution used was adjusted in each case so that the final excess over the NaOH was in all cases 0·75 N. Curve 1, 1·5 N-NaOH; curve 2, 0·75 N-NaOH; curve 3, 0·38 N-NaOH; curve 4, as 3 except that the reaction was carried out at 15° instead of 22°.

that at 0·38 N the values obtained are almost constant over reaction periods from 4 to 7 min. With aminoacetone, by contrast (Fig. 3), reduction of the NaOH concentration results in lower colour yields, and this cannot be compensated by increasing the reaction time, since destruction of the colour-forming complex proceeds rapidly irrespective of the NaOH concentration.

Effect of temperature. As described above, the colour yields obtained from ALA reached a stable plateau value over a reaction period of 4–7 min. (Fig. 2, curve 3) when 0·38 N-NaOH was present and this seemed to offer the possibility of a suitable procedure for ALA assay. However, Fig. 2 (curves 3 and 4) shows that reduction of the temperature from 22° to 15° alters the course of the reaction so that accurate estimations are impossible without temperature control. It is also clear from Fig. 3 that estimation of aminoacetone at room temperature is impracticable. The effect of carrying out the reaction at 0° was therefore studied. Fig. 4 shows that at this temperature, in the presence of 0·75 N-NaOH, stable plateau values are obtained from ALA after reaction periods of 14–16 min.; similarly, colour intensities obtained with aminoacetone are almost constant after reaction periods of 5–7 min. Under these conditions of estimation, errors due to inaccuracies in timing are negligible.

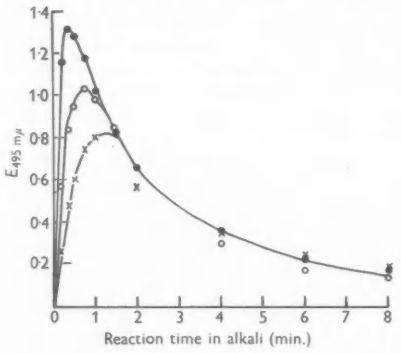

Fig. 3. Effect of reaction time in alkali on the colour yield from aminoacetone at different concentrations of NaOH. Conditions as in Fig. 2 except that samples contained 1·0 μmole of aminoacetone. ●, 1·5 N-NaOH; ○, 0·75 N-NaOH; ×, 0·38 N-NaOH.

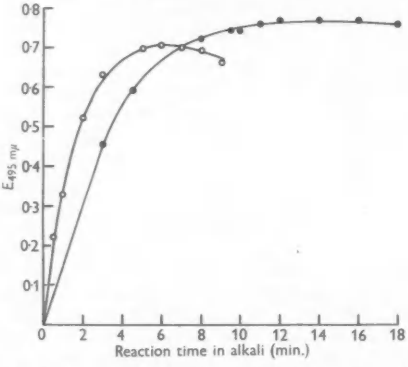

Fig. 4. Colour production from ALA (●) and aminoacetone (○) at 0°. 0·5 μmole samples of the amino ketones in 2·5 ml. volume were cooled to 0° in an ice–water bath and 0·5 ml. of 5 N-NaOH, previously cooled to 0°, added. At the appropriate times 1·75 ml. of 4 N-HCl was added and colours were read after 10 min. at room temperature against a reagent blank.

Method for estimation of δ-aminolaevulic acid and aminoacetone. The procedure finally adopted for estimation of the amino ketones is as follows: the sample (0·05–0·8 μmole of ALA or 0·05–0·5 μmole of amino acetone) in a volume of 2·5 ml. is mixed with 0·25 ml. of a solution of picric acid saturated at room temperature and cooled to 0° in an ice–water bath. Sodium hydroxide (0·5 ml. of 5 N at 0°) is then added and, after mixing, the solution is allowed to stand at 0°. After a 6 min. or a 14 min. reaction period (aminoacetone and ALA determinations respectively), 1·75 ml. of 4 N-HCl is added, the solution mixed and brought to room temperature in a water bath. The absorption is read at 495 mμ after a further 10 min. period.

The absorption is proportional to the amino ketone concentration up to 0·5 μmole for aminoacetone and to 0·8 μmole for ALA (Fig. 5). Readings of 0·76 and 0·71 are obtained with 0·5 μmole of ALA and aminoacetone respectively.

Errors in pipetting the reagents have negligible effects on the determination. A 20 % reduction in NaOH concentration reduces colour yield by only 6 % and a 20 % increase has no effect. Variations of 20 % in the amount of HCl added likewise have very small effects on the final colour intensity.

Colour stability and interfering substances. The colour produced with both amino ketones fades at the rate of 2 %/10 min. at room temperature.

Shuster (1956) has pointed out that many carbonyl compounds such as pyruvic and α-oxoglutaric acids and acetone produce colours in the picrate reaction but that these fade at room temperature. Under the conditions described above it has been found that 50 μmoles of α-oxoglutaric acid give an absorption corresponding to 0·16 μmole of aminoacetone and that 10 μmoles of oxoglutarate are equivalent to 0·08 μmole of aminoacetone. In most cases, therefore, keto acids will cause little interference with amino-ketone determinations, but if present in comparatively large amounts they

may be removed by a preliminary resin treatment. In practice the following treatment has been found satisfactory. The sample is passed through a column (5 cm. × 0·8 cm.) of Dowex 50 (H form) and the column washed with 20 bed-volumes of water. The amino compounds are then eluted with 25 ml. of 2N-HCl and the eluate evaporated to dryness *in vacuo*. The residue is then dissolved in a known volume of water and a suitable sample taken for determination of the amino ketone. Recoveries of aminoacetone (Table 2) and ALA (Table 1) after such treatment are essentially quantitative.

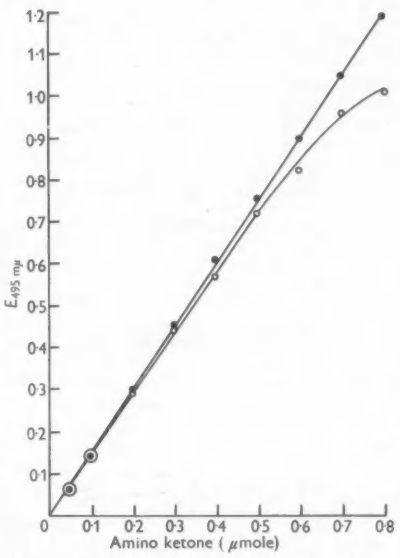

Fig. 5. Calibration curves for ALA (●) and aminoacetone (○) by the standard method. Colours were read against a reagent blank. ALA hydrochloride and aminoacetone toluene-*p*-sulphonic acid salts respectively were used as standards.

Glucose, threonine and inorganic phosphate (0·1 M) and 2·5% trichloroacetate were without effect on the colour production.

Recovery experiments. A homogenate was prepared by grinding 10 g. of rat liver in a Potter–Elvehjem apparatus with 10 ml. of 0·05 M-phosphate buffer, pH 7·0. Samples (3 ml.) of this homogenate were transferred to centrifuge tubes and varied amounts of ALA or aminoacetone added together with 1·0 ml. of 20% trichloroacetic acid. The volumes were adjusted to 5·0 ml. by the addition of water and the protein removed by centrifuging. Samples (2·0 ml.) of the supernatant liquid were adjusted to pH 3·0–6·5 by the addition of 5N-NaOH and ALA and aminoacetone determined by the procedure described above. The recoveries were too high by approximately 0·07 μmole irrespective of the amount of amino ketone present (Table 2); in view of this effect it would seem desirable to include internal standards in such determinations.

Separation of δ-aminolaevulic acid and aminoacetone

Ion-exchange resins. ALA and aminoacetone can be separated readily on the carboxylic acid resin Amberlite IRC-50 buffered at pH 4·7–5·0 with sodium acetate buffer. The column is equilibrated with the buffer, washed with water and the sample

Table 1. *Recovery of δ-aminolaevulic acid from a Dowex 50 column*

The ALA was contained in 20 ml. volumes of a mixture of 0·01 M-phosphate buffer (pH 7·0), 1% glucose, 0·1% NaCl and 0·01 M-glycine. The solution was treated on Dowex 50 columns as described in the text.

δ-Aminolaevulic acid (μmole)	
Added	Recovered
0·5	0·54
0·3	0·28
0·2	0·2
0·1	0·095

Table 2. *Recovery experiments from rat-liver homogenates*

Experimental details are described in the text. Figures are corrected for the blank values in homogenate alone.

	μmoles		
Amino ketone	Added	Recovered	Error
δ-Aminolaevulic acid	1·0	1·075	+0·07
δ-Aminolaevulic acid	0·5	0·575	+0·07
δ-Aminolaevulic acid	0·25	0·29	+0·04
Aminoacetone	1·0	1·07	+0·07
Aminoacetone	0·5	0·58	+0·08
Aminoacetone	0·25	0·32	+0·07
Aminoacetone (Dowex 50-treated)	1·0	1·11	+0·11
Aminoacetone (Dowex 50-treated)	0·5	0·58	+0·08
Aminoacetone (Dowex 50-treated)	0·25	0·32	+0·07

at pH 5·0 run slowly through, when aminoacetone is retained and ALA runs out. After the column has been washed with water the aminoacetone is eluted with HCl. For subsequent chromatography the eluate is evaporated to dryness *in vacuo*, the aminoacetone hydrochloride extracted with absolute ethanol and the bulky precipitate of NaCl filtered off. The solution is then sufficiently salt-free for most purposes.

Paper chromatography. Chromatographic separation of ALA and aminoacetone can be achieved by using the following solvent system: ethanol–M-acetic acid–pyridine–water (95:10:3:3, by vol.) in which ALA and aminoacetone have R_F values of 0·15 and 0·3 respectively. In five acidic solvents tested, including butanol–acetic acid, ALA and aminoacetone had identical R_F values. In view of this, and particularly since alkaline solvents cannot be used, the two compounds could easily be confused. Both give the same colour reactions with ninhydrin (yellow, turning purple at room temperature). ALA and aminoacetone can be separated by paper electrophoresis at pH 4·0–6·5 and the two can also be distinguished chromatographically by first converting them into pyrroles (Mauzerall & Granick, 1956).

SUMMARY

Conditions for the determination of δ-aminolaevulic acid and aminoacetone by the picrate reaction have been investigated and a simple procedure has been developed for the estimation of 0·05–0·8 μmole of δ-aminolaevulic acid or 0·05–0·5 μmole of aminoacetone. Methods for separating the two compounds are also described.

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The Chromatography of L-Myosin on Diethylaminoethylcellulose

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The methods employed by most workers for the purification of L-myosin from extracts of whole muscle usually involve two main principles. These are the selective precipitation at ionic strength about 0·3 of any actomyosin which may be present and the removal of albumin and other protein impurities by repeated precipitation by dilution with water to an ionic strength at which the myosin itself is insoluble (Edsall, 1930; Bailey, 1942; Szent-Györgyi, 1945; Portzehl, Schramm & Weber, 1950; Mommaerts & Parrish, 1951; Perry, 1955). Salting-out procedures have also been employed, although when ammonium sulphate is used (Tsao, 1953) appreciable loss in adenosine triphosphatase activity occurs.

Even in the best preparations of L-myosin from rabbit skeletal muscle, identifiable impurities, such as 5′-adenylic deaminase (Summerson & Meister, 1944) and nucleic acid (Bailey, 1946; Lajtha, 1951), are known to be present. In addition, on electrophoretic and ultracentrifugal analyses of such preparations it is often possible to detect small amounts of substances which have not been definitely characterized (Mommaerts & Parrish, 1951; Tsao, 1953; Raeber, Schapira & Dreyfus, 1955).

In view of the ability of myosin to interact with a number of proteins and of the limitations inherent in the standard methods of establishing protein homogeneity when applied to a substance with a molecular asymmetry comparable with that of myosin, it is very difficult to decide by the usual criteria just how homogeneous the usual preparations of L-myosin are. Certainly on electrophoretic evidence alone some workers consider the standard L-myosin preparations to contain 10–15% of impurities (Mommaerts & Parrish, 1951; Tsao, 1953). This is perhaps not surprising in view of the relative lack of specificity of the methods employed in the isolation of myosin, for it is a somewhat labile protein and the more selective methods of preparation are not readily applied. The use of synthetic ion-exchange resins has been limited to the chromatography of the more robust proteins, and until now there has been no report of the

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successful study of myosin by this technique. Diethylaminoethylcellulose (Peterson & Sober, 1956), which has achieved considerable success as a relatively-mild chromatographic medium effective in the region pH 7–8 for the separation of proteins, offered some promise. This paper deals with its application to the chromatography of myosin. It forms part of a general study of the muscle proteins by ion-exchange chromatography (Perry & Zydowo, 1959*a*, *b*).

METHODS

Chromatography. Diethylaminoethylcellulose and carboxymethylcellulose were prepared by the methods of Peterson & Sober (1956) and chromatography was in general carried out as described by Perry & Zydowo (1959*a*, *b*). Myosin solutions were equilibrated against the appropriate buffer by dialysis, and centrifuged for 20 min. at 10 000 *g* before application to the column, which had also previously been fully equilibrated with buffer. Usually the $E_{1\,cm.}$ (280 mμ) of the myosin solutions was within the range 3–4; occasionally at pH 7·6 somewhat more concentrated solutions [$E_{1\,cm.}$ less than 8·0 at 280 mμ] were applied. It was not practicable to apply more concentrated myosin solutions with the grade of diethylaminoethylcellulose used, which was made from Solka-Floc BW. 200 (Brown Co., Boston, Mass., U.S.A.), for the rate of entry into the column became very slow and the flow tended to stop completely. All chromatographic procedures and preparations of protein solutions were carried out at 1–2°.

L-Myosin. This protein was prepared as described by Perry (1955). No significant fall in viscosity was obtained on addition of adenosine triphosphate (ATP) to this preparation, indicating the absence of actin.

L-Myosin solution could be further purified by chromatography at 0° on diethylaminoethylcellulose as follows. A solution of $E_{1\,cm.}$ 3–4 at 280 mμ was equilibrated by dialysis against 0·16M-KCl–20 mM-tris-chloride buffer (pH 8·1) and run into a column (15–20 cm. × 2 cm. diam.) of diethylaminoethylcellulose equilibrated against the same buffer (2–5 mg. of protein N/g. of diethylaminoethylcellulose). After application of the myosin the column was washed with the original buffer solution until E_{280} of the eluate was negligible. Myosin was then eluted by the direct application of 0·22M-KCl, 20 mM-tris–HCl (pH 8·1). It was precipitated from the eluate by dialysis against 0·04M-KCl.

Enzymic assays. Adenosine triphosphatase (ATPase) assays were carried out at 25° as described by Perry & Grey (1956). The final incubation mixture of total volume 2 ml. usually contained: 5 mM-ATP; 5 mM-$CaCl_2$; 50 mM-tris–HCl buffer (pH 7·6); 0·3 ml. of 0·25M-KCl, 5 mM-tris–HCl (pH 7·6), or eluted myosin in the same solution; KCl to bring the final concentration to 0·2M. In some cases, for more accurate determinations, assays were carried out at three different concentrations of enzyme from a given fraction and specific activities calculated from the straight-line graphs obtained by plotting phosphate liberated against amount of protein. Specific ATPase activities were expressed as μg. of phosphorus liberated in 5 min. by 1 ml. of protein solution of $E_{1\,cm.}$ 1·0 at 280 mμ.

5′-Adenylic deaminase activity was determined as described by Perry & Zydowo (1959*a*).

Ribose and phosphorus determinations. The total phosphorus content of myosin preparations was determined by the method of Fiske & Subbarow (1925). For nucleic acid ribose and phosphorus estimations myosin solutions were precipitated at 0° with an equal volume of 15% (w/w) trichloroacetic acid, and the residue was extracted as described by Perry & Zydowo (1959*b*). Estimations were made on the extract obtained with hot 10% (w/v) $HClO_4$.

pH *measurements.* pH was measured on the buffers diluted to the concentrations used with the Pye glass electrode. The electrode was standardized at pH 6·99 with Soloid standard buffer tablets (Burroughs Wellcome and Co.).

RESULTS

Ion-exchange chromatography of myosin on cellulose derivatives presents certain difficulties because of the limited range of pH and ionic strength over which this protein is stable and soluble. For example at pH 5 and below the ATPase activity falls off rapidly, and at pH 7·0 an ionic strength of about 0·25 is necessary to keep appreciable amounts of myosin in solution.

In previous work (Perry & Zydowo, 1959*a*, *b*) it was convenient to elute the muscle proteins from diethylaminoethylcellulose by increasing the salt concentration at a constant pH. At pH 7·6 and below it was not practical, however, to employ this procedure with myosin, for when the protein dissolved in 0·25M-KCl containing 5 mM- or 20 mM-tris–HCl buffer, pH 7·6, was applied to a column of diethylaminoethylcellulose it was not held, although it appeared to be somewhat retarded on passing through the column. At lower ionic strengths the solubility of myosin decreases, and preliminary experiments in which the myosin was applied in 0·23M-KCl, 5 mM-tris–HCl (pH 7·6) were discontinued as only part of the myosin passed through unheld. Part appeared to be held or precipitated on the cellulose and was subsequently not satisfactorily eluted when the KCl concentration reached values at which, if used for the initial application to the cellulose, the myosin would not have been held at all. In 0·25M-KCl, 5 mM-glyoxaline–HCl buffer (pH 7·1) myosin was likewise not held, but if it was applied in 0·25M-KCl, 5 mM-histidine–HCl (pH 6·7) only part of the protein passed through unheld and elution of this material was impracticably slow.

A single attempt was made to chromatograph myosin on carboxymethylcellulose at pH 4·3. In 25 mM-sodium acetate buffer at this pH myosin is soluble but its ATPase activity is much reduced. Apart from a small amount of protein which was not held when myosin dissolved in the buffer was applied to the carboxymethylcellulose column, the bulk of the protein was strongly held and not eluted by M-KCl at this pH.

When myosin in 0·25M-KCl, 5 mM-tris–HCl (pH 7·6) was applied to a column of diethylaminoethylcellulose, about 40 cm. by 1 cm. diameter, and subsequently eluted by further application of the same buffer, the appearance of the eluted peak is shown in Fig. 1. Usually the peak was somewhat irregular in form, its appearance suggesting that it was composite, and often discrete steps could be recognized on the trailing side. With shorter and wider columns the peak was more compact but the same features were usually present. The variation in appearance of the peak appeared to depend more on the sample of diethylaminoethylcellulose and the column made from it than on the myosin preparation itself. For example, the chromatograms illustrated in Figs. 1 and 5 were obtained with the same sample of myosin. Although similar features can be recognized in both, the resolution is better in the experiment illustrated in Fig. 1.

Where myosin was chromatographed into a broad irregular peak, the behaviour of fractions on rechromatographing was determined by their original position on the peak. Material from the front of the peak, e.g. from fraction *A* in Fig. 2, on re-running on the same column under the same conditions underwent little or no retardation. Myosin from the trailing edge of the peak (fraction *B*, Fig. 2) was retarded compared to fraction *A*, however, when chromatographed again under identical conditions. This experiment indicated that fractions with real differences in chromatographic behaviour were present.

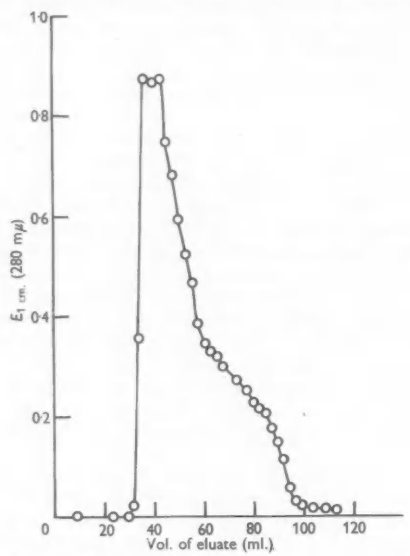

Fig. 1. Chromatography of L-myosin on diethylaminoethylcellulose. Protein solution (5 ml.; $E_{1\ cm.}$ 7·49 at 280 mμ) in 0·25M-KCl, 5 mM-tris–HCl (pH 7·5) was applied to a column 45·5 cm. × 1 cm. diameter and eluted with the same buffer.

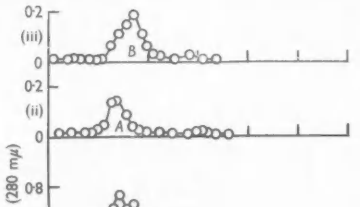

Fig. 2. Rechromatography of L-myosin eluted from diethylaminoethylcellulose. (i) 5 ml. of myosin ($E_{1\ cm.}$ 4·96 at 280 mμ) in 0·25M-KCl, 5 mM-tris–HCl (pH 7·6) was applied to a column 40 cm. × 1 cm. diameter, and eluted with the same buffer. Fractions *A* and *B* were concentrated by precipitation, by dialysis against 0·04M-KCl. (ii) 1·9 ml. of fraction *A* in 0·25M-KCl, 5 mM-tris–HCl (pH 7·6) was re-run on the column used for (i). (iii) 2·1 ml. of fraction *B* in 0·25M-KCl, 5 mM-tris–HCl (pH 7·6), was re-run on the same column as was used for (i) and (ii).

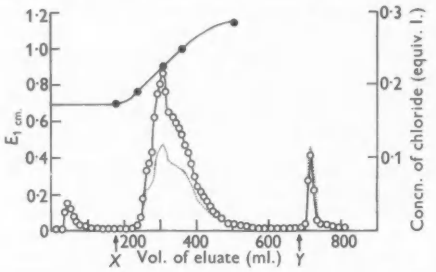

Fig. 3. Chromatography of L-myosin on diethylaminoethylcellulose. Protein solution (35 ml.; $E_{1\ cm.}$ 5·52 at 280 mμ) in 0·16M-KCl, 20 mM-tris–HCl (pH 8·2) was applied to a column 14 cm. × 2 cm. diameter. Elution by gradient to 0·3M-KCl, 20 mM-tris–HCl (pH 7·2) was applied at point *X* and step to 1·5M-KCl, 20 mM-tris–HCl (pH 8·2) at point *Y*. ○, $E_{280\ m\mu}$; ●, concentration of chloride; - - -, $E_{260\ m\mu}$.

By increasing the pH to 8·0–8·2 with 5 mM–20 mM-tris–HCl buffer satisfactory solutions of freshly prepared myosin ($E_{1\,cm.}$ 3·0–4·0 at 280 mμ) could be obtained in 0·16 M-KCl. Samples of myosin which had been stored 7–14 days were frequently less soluble in this solution than was freshly prepared material. Under these conditions myosin was held on the column and could be eluted when a gradient was applied (Fig. 3). On some occasions a small amount of material having a high 5′-adenylic deaminase activity, but without ATPase activity, passed through the column before the gradient was applied. More often, however, relatively small amounts of material with ATPase activity passed unheld through the column with the deaminase-rich material. The reason for this was not clear but it was apparently not due to overloading of the column. The peak with ATPase activity either appeared just behind that rich in 5′-adenylic deaminase activity or it was not distinguishable from it. The main peak which was obtained on application of a KCl gradient possessed ATPase activity and varied somewhat in shape according to the volume required for its elution. Its shape was usually similar to that illustrated in Fig. 3 and resembled the peak obtained when chromatography was carried out in 0·25 M-KCl, 5 mM-tris–HCl, pH 7·6. The peak, which was never symmetrical, occurred at a chloride concentration of 0·20–0·22 equiv./l.

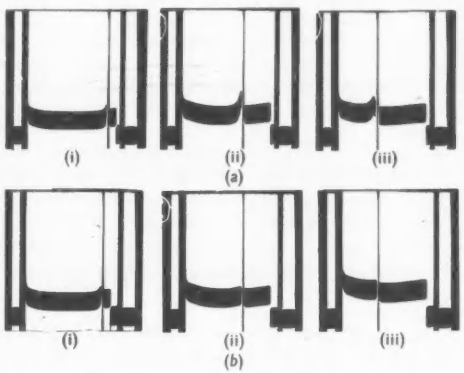

Fig. 4. Ultracentrifuge diagrams of myosin before and after chromatography on diethylaminoethylcellulose. Conditions were: 0·25 M-KCl, 0·05 M-potassium phosphate buffer (pH 7·2); $5{\cdot}98 \times 10^4$ rev./min.; 20°. (*a*) L-myosin was applied in 0·16 M-KCl, 20 mM-tris–HCl (pH 8·2) and eluted by application of gradient to 0·3 M-KCl; (i) 16 min., (ii) 64 min., (iii) 112 min. (*b*) The same sample of L-myosin was equilibrated with 0·16 M-KCl, 20 mM-tris–HCl (pH 8·2), but not chromatographed; (i) 16 min., (ii) 64 min., (iii) 112 min.

Myosin which had been chromatographed on diethylaminoethylcellulose retained its ability to combine with actin to form actomyosin, as indicated by a fall in viscosity on the addition of ATP to a solution in which both proteins were present (kindly carried out by Mr L. Leadbeater). The specific ATPase activity was appreciably increased and, in so far as the chromatographed protein was insoluble at pH 7·0 in 0·04 M-KCl and soluble in 0·5 M-KCl, the solubility characteristics were unchanged. On electrophoresis in 0·25 M-KCl, 50 mM-potassium phosphate buffer (pH 7·2) for 10 hr., no significant difference between untreated myosin and that which had been chromatographed at pH 7·6 or 8·2 could be detected. (At pH 8·2 electrophoresis was carried out on the main ATPase-rich peak.) At both pH values the myosin moved as a single peak which was somewhat asymmetric, and although it remained extremely sharp throughout a prolonged electrophoresis the descending boundary displayed minor irregularities which could be interpreted as evidence of heterogeneity.

In the ultracentrifuge the chromatographed myosin presented, in addition to the sharp characteristic myosin boundary, a slightly faster broader boundary which was not evident in control samples of unchromatographed myosin (Fig. 4).

Adenosine triphosphatase activity of myosin chromatographed on diethylaminoethylcellulose. The curve obtained by plotting the ATPase activity per ml. of the chromatographed myosin against the volume of eluent at both pH 7·6 and 8·1 followed in a general way a similar plot of absorption at 280 mμ. If, however, the activity was calculated as specific activity (see Methods) then the following results were obtained:

(1) The specific activity was not constant along the eluted peak. In general two main peaks could be seen, as illustrated in Fig. 5. The first peak of specific activity was close to or slightly in front of the main 280 mμ-absorbing peak, whereas the second usually occurred in the trailing edge. Occasionally the first peak of specific activity split into two, but the most persistent features were the fractions of lower specific activity obtained when about half, or slightly more than half, of the myosin had been eluted from the column. The data from a number of experiments are summarized in Table 1.

(2) In some preparations the specific activity at the front of the E_{280} peak was low, suggesting that inactive myosin or non-myosin material, which was not retarded by the cellulose, was present. This was probably due to the material without ATPase activity, but rich in 5′-adenylic deaminase activity, which was eluted as a separate peak with 0·16 M-KCl, 20 mM-tris–HCl (pH 8·2) (see above).

(3) With the exception of the small amount of material which was sometimes observed at the

front of the peak, the specific activity was greater at all other points along the peak at which it was measured than that of the original myosin (Table 1).

These observations were all made on myosin eluted at constant ionic strength, i.e. with 0·25 M-KCl, 5 mM-tris–HCl (pH 7·6). Limited studies on myosin applied to diethylaminoethylcellulose in 0·16 M-KCl, 20 mM-tris–HCl (pH 8·1), suggested that there was a similar distribution of specific ATPase activity along the myosin peak eluted on application of a gradient.

The increase in specific activity related to E_{280} could not be explained by the preferential removal of ribonucleic acid (see below), which has a relatively greater $E_{1\,cm.}$ at 280 mμ than has protein. Comparison of the absorption at 280 mμ per mg. of protein nitrogen before and after chromatography on diethylaminoethylcellulose indicated a fall of less than 3 %. This finding, and control experiments in which ATPase activity was related directly to the nitrogen content of the fractions, clearly showed that after chromatography there was a significant increase in actual specific ATPase activity.

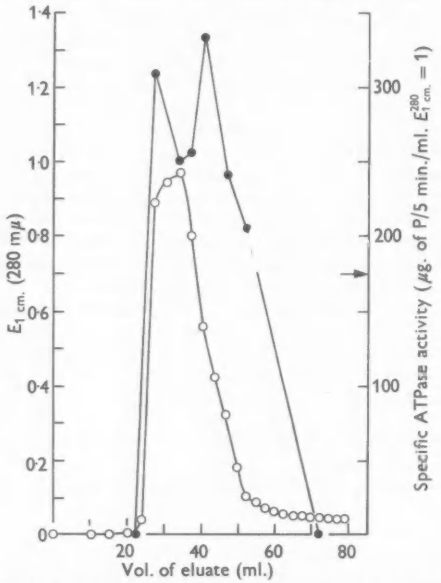

Fig. 5. Specific ATPase activity of L-myosin after chromatography on diethylaminoethylcellulose. Protein solution (4 ml.; $E_{1\,cm.}$ 7·46 at 280 mμ) in 0·25 M-KCl, 5 mM-tris–HCl (pH 7·6), was applied to a column 40 cm. × 1 cm. diameter and eluted with the same buffer. ○, $E_{280\,mμ}$; ●, specific ATPase activity. The arrow on the specific activity ordinate indicates the activity of unchromatographed myosin.

5′-Adenylic deaminase activity. The 5′-adenylic deaminase activity of the chromatographed myosin was much less systematically studied than was the ATPase activity. Preliminary experiments indicated that both halves of the main peak eluted at pH 7·6 possessed deaminase of activity comparable, although not identical, with that of the original myosin. Further work was not done at this pH, as from an earlier investigation (Perry & Zydowo, 1959*a*) it appeared that 5′-adenylic deaminase was probably eluted from diethylaminoethylcellulose at a lower ionic strength than that required to elute myosin. For this reason chromatography at pH 8·2, at which the myosin could be held on the diethylaminoethylcellulose, was considered more likely to achieve separation of the two enzymes. The small peak without ATPase activity, which was eluted by 0·16 M-KCl, 20 mM-tris–HCl buffer (pH 8·2), possessed deaminase activity 5–10 times that of the original myosin. When the material which passed unheld through the column before application of the gradient also possessed ATPase activity, the specific activity with regard to ATPase was usually lower than that of unchromatographed myosin, whereas the specific activity with regard to 5′-adenylic deaminase was appreciably higher. The main myosin peak obtained on

Table 1. *Specific adenosine triphosphatase activity of fractions of L-myosin eluted from diethylaminoethylcellulose*

Myosin was applied in 0·25 M-KCl, 5 mM-tris–HCl, pH 7·6, and eluted with same buffer, except in the case of Expt. 14 which was carried out in 0·25 M-KCl, 5 mM-glyoxaline–HCl buffer (pH 7·1). Specific activity was measured at 25° = μg. of phosphorus/5 min./1 ml. of protein, $E_{1\,cm.}$ 1·0 at 280 mμ.

Expt. no.	Myosin preparation	Age (days)	Specific ATPase activity: Unchromatographed	Chromatographed: 1st peak	Chromatographed: 2nd peak	Chromatographed: Minimum between peaks
8	A	11	179	232	294	188
13	B	13	161	303	229	198
14	B	16	175	255	235	218
15	B	22	176	309	335	252
19	C	4	186	235	222	210

Table 2. *Phosphorus and ribonucleic acid content of L-myosin preparations before and after chromatography on diethylaminoethylcellulose*

Results are expressed in μg./g. of myosin N. Those in columns 2 and 4 are obtained from the analysis of hot 10% $HClO_4$ extract of myosin preparations (see Methods). RNA phosphorus is obtained from the ribose figures assuming that 53·1% of total ribose is estimated under the condition of the orcinol reaction (Perry & Zydowo, 1959*b*).

		(1) Total P	(2) Ribose	(3) RNA-P	(4) Total P in $HClO_4$ extract
Expt. 1.	Before chromatography	1608	3375	1312	1111
	After elution by 0·25 M-KCl, 20 mM-tris (pH 7·6)	104	280	109	53
Expt. 2.	Before chromatography	1091	2020	785	651
	After elution by KCl gradient in 20 mM-tris (pH 8·2)	153	49	19	45

subsequent application of a gradient had about one-third the deaminase activity of control unchromatographed samples. Thus chromatography on diethylaminoethylcellulose under these conditions partially removed the 5′-adenylic deaminase from L-myosin preparations.

Ribonucleic acid and phosphorus content of myosin. After complete elution of the myosin peak from diethylaminoethylcellulose columns to which the protein had been applied either in 0·25 M-KCl, 20 mM-tris–HCl (pH 7·6) or in 0·16 M-KCl, 20 mM-tris–HCl (pH 8·1), subsequent increase in the KCl concentration to 1·5 M eluted a further small peak. This peak differed from the other material eluted from the column in that the ratio E_{260}/E_{280} was usually greater than 1 (Fig. 5). It was probable that this fraction contained ribonucleic acid (RNA), since the material absorbed strongly at a maximum of about 258 mμ, was non-dialysable and relatively rich in pentose and phosphorus compared with myosin. The presence of protein was evident from the general properties of this fraction; also it gave a positive biuret test and the minimum of the ultraviolet-absorption spectrum was at 240 mμ, rather than 230 mμ which is characteristic of pure RNA (Beaven, Holiday & Johnson, 1955). On dialysis against distilled water almost all the material absorbing at 280 and 260 mμ was precipitated. In this property the fraction differed from the nucleoprotein isolated from the myofibril (Perry & Zydowo, 1959*b*). Nevertheless the RNA-containing fraction from myosin probably contained this myofibrillar ribonucleoprotein, together with small amounts of myosin, whose presence was indicated by ATPase activity (usually less than 20% of that of myosin of comparable extinction at 280 mμ). Tropomyosin, which often contaminates myosin preparations, would also be expected to be eluted with this fraction unless special steps were taken to elute it from the column after removal of the myosin and before the fraction containing RNA is displaced (Perry & Zydowo, 1959*a*).

L-Myosin preparations used in this investigation contained as a persistent impurity 0·2–0·3% of RNA, values that are somewhat less than those reported by Mihalyi, Brodley & Knoller (1957). Myosin which had passed through a diethylaminoethylcellulose column, whether it was held or not, i.e. at pH 8·1 or 7·6, contained little or no RNA (see Table 2). It appeared therefore that the RNA-containing fraction eluted at higher concentrations of KCl represented almost all the RNA originally present in myosin preparations. The total phosphorus content of myosin was reduced by chromatography under these conditions to about 0·002%, a barely significant figure and equivalent to less than 5–10% of the original value.

The value for RNA phosphorus was calculated from the ribose figures assuming that 53·1% of the total ribose of the RNA, i.e. the value obtained for the myofibrillar nucleoprotein (Perry & Zydowo, 1959*b*), was estimated under conditions of the orcinol reaction. For the unchromatographed myosin these values were, however, higher than the estimated total phosphorus of the 10% $HClO_4$ extracts, suggesting that either the factor was too high or ribose other than that present in RNA was present in the 10% $HClO_4$ extracts. Further, as not all the total phosphorus was extracted from unchromatographed myosin by $HClO_4$, part may have been due to other substances, e.g. phospholipid. Much of this phosphorus insoluble in 10% $HClO_4$ was also removed by chromatography on diethylaminoethylcellulose.

DISCUSSION

The use of diethylaminoethylcellulose offers a means of applying chromatographic procedures for the study of myosin. Since the chromatographed myosin had a higher specific ATPase activity than the original preparation and retained its ability to form actomyosin, no loss in the biological activity of the protein was detectable. It is apparent that L-myosin prepared by conventional methods contains substances which do not possess ATPase activity. Chromatography provides a simple method

of removing at least one of these impurities, an RNA-containing fraction which is responsible for the bulk of the phosphorus present in unchromatographed myosin preparations. The RNA is associated with protein and part of this complex is probably identical with the ribonucleoprotein which can be isolated from the myofibril (Perry & Zydowo, 1959*a*, *b*). Traces of phosphorus remained associated with myosin after chromatography, but the amounts estimated were close to the limits of accuracy of the methods used. It is considered that this small amount of phosphorus is present as a contaminant and does not function as the prosthetic group of myosin.

The shape of the main peak which constitutes the bulk of the eluted material suggested heterogeneity under almost all the conditions of chromatography tried. Further evidence of heterogeneity is provided by the fairly regular pattern of variation of specific ATPase activity along the main peak of eluted material and by re-running fractions of chromatographed myosin on diethylaminoethylcellulose. When the retarded myosin fraction (fraction *B*, Fig. 2) was run alone its elution volume was lower than when the whole myosin was chromatographed. This may be due to displacement effects by the other myosin fractions. The simple increase of ATPase activity after chromatography could be explained by the active renewal either of some inhibitor or of enzymically inert material. At pH 7·6 usually 67–87 % of the applied protein, as judged by absorption at 280 mμ, was recovered in the main eluted peak.

Myosin ATPase is known to be sensitive to variations in the ionic nature of the assay medium; such effects cannot be used to explain the variation in specific activity along the eluted peak, for care was taken to ensure that assays were carried out under standard conditions. One possible explanation is that there are several different forms of myosin, with different enzymic activity. These forms either could be present in the original myosin or could be produced by modification of the original protein as a consequence of chromatography on the diethylaminoethylcellulose. This investigation provides no clear evidence by which to decide between these possibilities. Changes in growth hormone during chromatography on diethylaminoethylcellulose have been reported by Ellis & Simpson (1956) and there is evidence from the present study of a difference in the sedimentation behaviour of chromatographed myosin compared with the untreated protein. This change in sedimentation pattern may represent partial aggregation of the myosin. It has been reported, however, that such changes occur spontaneously on storage of myosin (Holtzer, 1956), and it is not easy to relate them to the distribution of specific ATPase activity along the myosin peak. Further work is needed to interpret these changes in sedimentation pattern.

Another possible explanation is that normal preparations of L-myosin contain appreciable amounts of enzymically inactive material which is closely associated with the ATPase-containing protein and coprecipitated with it. The findings reported here could be explained if during elution from diethylaminoethylcellulose some separation of such enzymic and non-enzymic components occurred so that the component devoid of enzymic activity became concentrated mainly, although perhaps not entirely, about the centre or second half of the eluted material, the ATPase itself being eluted as a fairly broad peak over the whole range. Treatment of myosin with proteolytic enzymes of different specificities splits myosin into similar enzymic and non-enzymically active fragments, the H- and L-meromyosins (Szent-Györgyi, 1953; Gergely, Gouvea & Kariban, 1955). Several workers have considered the possibility of the composite nature of myosin (Tsao, 1953; Kominz, Hough, Symonds & Laki, 1954; Snellman, 1956; Kominz, Carroll, Smith & Mitchell, 1959). It is conceivable that the conventional preparations could consist of a firmly bound complex of two or more components, not involving covalent bonds, whose separation is facilitated by slight modification of the components, e.g. by proteolytic enzymes (cf. Middlebrook, 1958). Evidence which suggests that the meromyosins may lead an independent existence is supplied both from studies on their turnover *in vivo* (Velick, 1956) and from investigations with fluorescent antibodies (Holtzer & Marshall, 1958) on the localization of myosin and the meromyosins in the myofibril.

Whatever may be the explanation of the enzymic results, chromatography on diethylaminoethylcellulose offers an effective way of removing certain impurities in myosin and gives results which suggest that the conventional preparations of this protein may be complex systems. The findings of a number of physical and biochemical investigations on L-myosin may have to be reconsidered in the light of this apparent complexity. For example, if the ribonucleoprotein contaminant plays a part in protein synthesis in muscle, it has possibly a relatively high turnover rate, thus influencing appreciably the turnover values which have been obtained with conventional myosin preparations in which it is present as an impurity.

SUMMARY

1. The chromatographic behaviour of L-myosin on diethylaminoethylcellulose has been investigated at pH 7·6 and 8·2.

2. Evidence is presented for a regular variation in the specific adenosine triphosphatase activity of myosin fractions eluted from diethylaminoethylcellulose.

3. Partial separation of 5′-adenylic deaminase activity has been obtained by chromatography at pH 8·2.

4. Myosin has been prepared with less than 0·002% of phosphorus and virtually free from the ribonucleic acid invariably present in L-myosin preparations.

I am grateful to Miss F. Johnson and Mr T. C. Grey for skilled technical assistance at various times during this investigation. My thanks are also due to Dr R. R. Porter for a gift of diethylaminoethylcellulose and to Mr B. Boon and Mr B. Slater for help with the Tiselius and ultracentrifuge experiments. This investigation was in part supported by a research expenses grant from the Medical Research Council.

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Distribution and Activity of Monoamine Oxidase in Mouse Tissues

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During a recent study of the radioprotective action of 5-hydroxytryptamine in the mouse (Hope, 1959) we were impressed by its low toxicity in this species compared with the rat. The LD_{50} for 5-hydroxytryptamine administered intraperitoneally as the creatinine sulphate in the mouse was approximately 1 g./kg. body weight. Rapport & Virno (1952) found that the LD_{50} for this substance administered in the same way in rats was 50 mg./kg. body weight, i.e., it is 20 times as toxic to the rat as to the mouse.

Rapport & Virno (1952) investigated in a number of mammalian species the fall in oxygen consumption after the administration of 5-hydroxytryptamine. The rat was more sensitive in this respect than any other species examined. The authors suggested that the low amine oxidase activity of rat kidney was responsible.

In view of the now well-established fact that amine oxidase is an important catalyst of the destruction of 5-hydroxytryptamine in mammals (Blaschko, 1958) we decided to study the activity of the enzyme in mouse kidney and some other mouse tissues.

In this paper the rate of oxidation of 5-hydroxytryptamine has been compared with that of representatives from the main types of monoamine oxidase substrates. These included tryptamine, β-phenylethylamine, *iso*amylamine and benzylamine. Tyramine, although in some respects an unsatisfactory substrate of amine oxidase (Blaschko & Himms, 1954), was also included in order to

compare the results obtained with those of other workers.

The amine oxidase of mouse kidney has been shown to have a high degree of specificity for 5-hydroxytryptamine; this amine was the most rapidly oxidized substrate. This finding was followed by a study of four other tissues: brain, liver, lung and spleen. The presence of an amine oxidase in mouse liver has previously been described by Kobayashi (1957). This enzyme possesses many of the properties of monoamine oxidase yet it also attacks histamine and cadaverine. Davison, Lessin & Parkes (1957) have demonstrated the presence of tyramine oxidase in mouse liver and brain. Brain amine oxidase is worthy of special note because of the current interest in the functions of amines in this organ.

The effects of the amine oxidase inhibitors amphetamine [(+) and (−)] and iproniazid on the enzymic activity of mouse tissues have been investigated. Use has been made of homogenates of brain tissue from mice treated with iproniazid in a study of the spontaneous oxidation of 5-hydroxytryptamine during the determination of the optimum substrate concentration for this compound.

Since guanidines are known to be inhibitors of monoamine oxidase an attempt to produce an inhibitor combining the high specificity of the 5-hydroxyindole residue with a guanidine was made. 5-Hydroxytryptacyamine, the guanidine analogue of 5-hydroxytryptamine, was synthesized and isolated as its creatinine sulphate. The inhibitory action of this substance on amine oxidase was measured.

MATERIALS AND METHODS

Substances. The amines *iso*amylamine, benzylamine and β-phenylethylamine as their hydrochlorides and 5-hydroxytryptamine creatinine sulphate monohydrate were obtained from Roche Products Ltd. Tryptamine and tyramine hydrochlorides were obtained from British Drug Houses Ltd.

The optical isomers of amphetamine hydrochloride were kindly given by Dr H. Blaschko. The resolution was effected by Professor P. Pratesi. We are also grateful to Dr A. L. Morrison (Roche Products Ltd.) for a gift of iproniazid phosphate.

Animals. The mice used were females of the R strain used in this Department [a cross between PCT♀ and (C3Hx 101)♂]. The animals were 14 weeks old when killed; their mean weight was 24·5 g.

Treatment with iproniazid. Iproniazid (*N-iso*nicotinoyl-*N'-iso*propylhydrazine) as the phosphate salt was administered intraperitoneally in a neutral 0·9% NaCl solution.

Preparation of tissue extracts. The animals were killed by a blow on the head followed by decapitation. Tissues from 30 or 40 animals were pooled and homogenized in an all-glass Potter–Elvehjem homogenizer with 4 vol. of 0·067 M-sodium phosphate buffer, pH 7·4. The homogenates were dialysed against distilled water (500 ml.) with three changes over a period of 18 hr. at 2° in order to reduce the blank consumption of O_2. This was usually 0·02–0·05 μl./mg. of fresh tissue/hr.

Measurement of enzymic activity. The enzymic activity was determined manometrically. The main compartment of the manometer flask contained 1 ml. of the dialysed homogenate and 0·6 ml. of 0·2 M-sodium phosphate buffer, pH 7·4. Where inhibitors were used, the buffer volume was reduced to 0·4 ml. and the inhibitor in a volume of 0·2 ml. was present in the main compartment. The system used for determining the rate of consumption of O_2 in the presence of cyanide was that devised by Krebs (1935). The side bulb contained 0·4 ml. of water or of 0·05 M-solution of the amine to be tested; the inner well contained 2 N-KOH and filter paper. The gas phase was O_2 and the temperature 37·5°.

The enzymic activity was expressed as q_{O_2}, i.e. as μl. of O_2 taken up/mg. of fresh tissue/hr.; it was calculated from the initial period when the rate of reaction was approximately constant.

5-Hydroxytryptamine (5-HT) was included as a substrate in each experiment and its rate of oxidation was compared with that of the other amines selected.

Preparation of the guanidine analogue of 5-hydroxytryptamine. 5-Hydroxytryptacyamine was synthesized from 5-HT creatinine sulphate by reaction with *S*-methyl-*iso*thiuronium sulphate. The reaction of amines with thiuronium salts for the synthesis of guanidines is well known.

5-HT creatinine sulphate monohydrate (1 g.) and *S*-methyl-*iso*thiuronium sulphate (2·19 g.) were dissolved in water (6 ml.). Ammonia was added until the mixture was strongly alkaline (3 ml. of aq. NH_3 soln., sp.gr. 0·880), and the temperature was maintained at 60–70° for 2 hr. The reaction occurred with evolution of methyl mercaptan. A further 2 ml. of aq. NH_3 soln. was added and the mixture boiled for 10 min. After acidification with 5 N-H_2SO_4 an equal volume of ethanol was added. A crystalline product rapidly separated in the cold. This product (0·65 g.) had m.p. 285–286°. Recrystallization was effected from water. The product separated in fine needles and had m.p. 287–289°. Elementary analysis showed that the product was 5-hydroxytryptacyamine creatinine sulphate (Found: C, 41·7; H, 5·57; N, 22·8; S, 7·35; $C_{15}H_{22}O_6N_7S$ requires C, 41·95; H, 5·40; N, 22·83; S, 7·47%). Paper chromatography in butan-1-ol–acetic acid–water (4:1:5; upper phase) on Whatman no. 4 paper gave a single spot having R_F 0·48. The position of the substance was revealed as a pink spot on the chromatograms by spraying with a mixture made as follows: diacetyl (1%, v/v, in water; 2·5 ml.) with α-naphthol (25%, w/v, in propan-1-ol; 20 ml.) was made up to 100 ml. with propan-1-ol. Just before use, 10 ml. of this solution, together with 5 ml. of 3 N-NaOH, was made up to 30 ml. with water to give the spray reagent. This system was developed from the method devised by Rosenberg, Ennor & Morrison (1956) for the estimation of guanidines.

RESULTS

The results of the substrate-specificity studies are summarized in Table 1. The result of one experiment with each substrate and tissue has been included. With brain and kidney the experiments

Table 1. *Relative rates of oxidation of 5-hydroxytryptamine and other amines by homogenates of mouse tissues*

Initial substrate concentration: 10 mM. Gas phase, O_2; temp., 37·5°. The q_{O_2} (µl. of O_2 consumed/mg. of fresh tissue/hr.) with 5-HT as substrate is given in parentheses.

Tissue	Substrate					
	5-Hydroxy-tryptamine	Tryptamine	Tyramine	β-Phenyl-ethylamine	*iso*Amylamine	Benzylamine
Brain	100 (0·490)	49	155	41	38	25
Liver	100 (0·695)	147	388	135	126	105
Kidney	100 (0·575)	47	77	26	0	0
Spleen	100 (0·248)	60·5	85	60·5	0	0
Lung	100 (0·750)	35	—	59	52	42

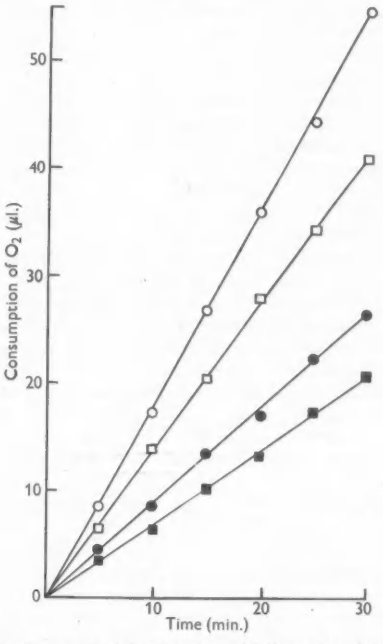

Fig. 1. Oxygen uptake due to oxidation of amines by mouse-kidney homogenate. Each flask contained: kidney homogenate equivalent to 110 mg. of fresh tissue (1 ml.); 0·2 M-sodium phosphate buffer, pH 7·4 (0·6 ml.). The substrates were present at initial concentrations of 0·01 M. Final volume, 2 ml. KOH was present in the centre well. Gas phase, O_2; temp., 37·5°. ○, 5-Hydroxytryptamine; ■, β-phenylethylamine; ●, tryptamine; □, tyramine.

have been repeated several times and the results confirmed. The activity of amine oxidase is constant in these two tissues. In four experiments with brain homogenates and 5-HT as substrate the q_{O_2} ranged from 0·46 to 0·49; for kidney the corresponding figures were 0·55 to 0·57.

Kidney. 5-HT was the amine most rapidly oxidized by homogenates of mouse kidney. Tryptamine, tyramine and β-phenylethylamine were also destroyed but at slower rates. *iso*-Amylamine and benzylamine do not appear to be substrates for the kidney enzyme; this distinguishes the kidney enzyme from those present in brain and liver. The kidney enzyme closely resembles the brain enzyme in the relative rates of oxidation of the other substrates, e.g. β-phenylethylamine was oxidized more slowly than tryptamine. The introduction of a hydroxyl group into the aromatic ring systems of these substances greatly increases the oxidation rates. Thus it can be seen in Fig. 1 that tyramine and 5-HT are the optimum substrates.

Brain. Homogenates of mouse brain oxidize 5-HT at about twice the rate for tryptamine or for β-phenylethylamine. The most rapidly oxidized substrate was tyramine. *iso*Amylamine and benzylamine were also oxidized, at slower rates.

Concentrations of 5-HT from 0·625 mM to 0·16 M were used to study the effect of substrate concentration on the reaction rate. The result of this experiment is shown in Fig. 2. The rate of oxidation increases linearly with the logarithm of the concentration of 5-HT up to 7·5 mM. At this point there is a discontinuity; the rate continues to increase but more slowly. The increase in rate beyond the discontinuity is due almost entirely to a spontaneous oxidation of 5-HT at high concentrations in an atmosphere of O_2. The lower curve in Fig. 2 shows the relationship between the concentration of 5-HT and the oxidation rate when homogenate prepared from the brains of mice treated with iproniazid was used. Two doses of iproniazid (100 mg./kg. body wt.) were given at 24 hr. intervals in order to inhibit fully the amine oxidase activity (see below). The homogenate from mice treated with iproniazid was used as a control because the rate of spontaneous oxidation of 5-HT is slightly higher in the absence of tissue proteins. The spontaneous rate cannot be measured manometrically when 5-HT concentrations below 0·01 M are used.

It seems reasonable to assume from this experiment that the optimum substrate concentration is approximately 0·01 M. The concentration for 50% of the maximum rate is 1·25 mM. This result may be compared with that obtained by Kohn (1937), who found the Michaelis constant for tyramine and the pig-liver amine oxidase to be 0·5 mM.

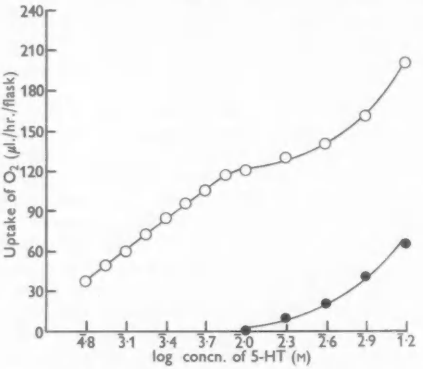

Fig. 2. Effect of 5-HT concentration on the oxidation rate with brain homogenates from normal (○) and iproniazid-treated (●) mice. Manometer flask contents: dialysed homogenate (1 ml.) derived from 300 mg. of fresh brain tissue; 0·2 M-sodium phosphate buffer, pH 7·4 (0·6 ml.); suitable substrate concentrations were present in the side arm to give the initial concentrations shown in the figure. Gas phase, O_2; temp., 37·5°.

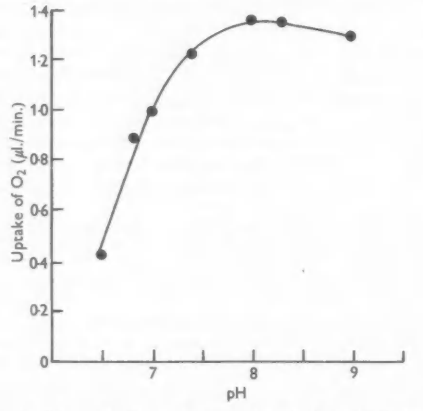

Fig. 3. Effect of pH on the rate of oxidation of 5-HT by mouse-brain homogenate. Each flask contained brain homogenate equivalent to 170 mg. of fresh tissue (1 ml.) and 0·2 M-sodium phosphate buffer of requisite pH (0·6 ml.). 5-HT was present in an initial concentration of 0·01 M. Final volume, 2 ml. Gas phase, O_2; temp., 37·5°.

In a similar experiment with tryptamine the rate of consumption of O_2 was constant when the substrate concentrations varied between 0·625 and 20 mM. This at first suggested that the optimum substrate concentration for tryptamine was lower than for 5-HT. However, when the reaction was taken to completion the consumption of O_2 was four times larger than could be expected from the following reaction scheme:

$$R{\cdot}CH_2{\cdot}NH_2 + O_2 + H_2O \rightarrow R{\cdot}CHO + H_2O_2 + NH_3$$
$$2H_2O_2 \rightarrow 2H_2O + O_2 .$$

Much of the extra oxygen consumption could be prevented by the addition of cyanide (10 mM) in the manometric experiments. This showed that enzymes other than the cyanide-insensitive amine oxidase were involved. The percentage inhibition of uptake of O_2 by cyanide was 10, 30 and 50 at tryptamine concentrations of 10, 5 and 2·5 mM respectively. The full significance of this result is not understood; it may be that the secondary oxidation of the product of amine oxidase action is sensitive to concentration. This complication rendered it impossible to estimate the Michaelis constant without purifying the enzyme.

The optimum pH for the brain enzyme was 8·1. The activity–pH curve is shown in Fig. 3.

Liver. In mouse-liver homogenates tyramine was the most rapidly oxidized substrate. The enzyme destroyed each of the substrates tested at appreciable rates; the lowest rate was observed with 5-HT. Thus the liver stands in marked contrast with the other tissues studied.

Other tissues. Lung and spleen tissue were also examined for enzymic activity. The highest q_{O_2} value for 5-HT oxidation was found with lung homogenates. 5-HT was moreover the most readily oxidized substrate.

Spleen tissue showed a relatively weak activity; 5-HT was again the most readily attacked substrate. Homogenates of spleen resembled those from kidney because neither *iso*amylamine nor benzylamine was oxidized.

A comparison of (+)- and (−)-amphetamine as inhibitor of amine oxidase in brain and kidney. 5-HT has been used as the substrate in a comparison of the optical isomers of amphetamine as inhibitors of monoamine oxidase activity in mouse-brain and kidney homogenates. A range of concentrations of the isomers was incubated with enzyme in the main compartment of the manometer flask during equilibration.

It can be seen in Fig. 4 that in the brain (+)-amphetamine was seven times as active as (−)-amphetamine. In kidney (+)-amphetamine was also the more active isomer but the difference observed was much less than that observed with the brain enzyme. (−)-Amphetamine is equally as

effective in the kidney as in the brain. The concentrations (mM) of the isomers necessary to inhibit the enzymic activity by 50% were:

	(+)-Amphetamine	(−)-Amphetamine
Brain	0·513	3·63
Kidney	1·51	3·63

Inhibition of amine oxidase by administration of iproniazid. Davison *et al.* (1957) found that the amine oxidase activity in brain and liver was completely inhibited in mice 24 hr. after the intraperitoneal administration of iproniazid (100 mg./kg. body wt.). The amine oxidase activity of the R strain of mice was more resistant to iproniazid than that of the albino mice used by Davison *et al.* After a second administration the brain enzyme became fully inhibited, yet the enzyme in the liver seemed not to be affected. From 10 to 20% of normal activity was retained with all substrates in liver. This was also true in kidney, except for β-phenylethylamine, where oxidation appeared to be fully inhibited by two injections of iproniazid. This may be due to an experimental error arising from the difficulty of measuring accurately rates of oxidation of the same order of magnitude as the blank O_2 consumption. The results of these experiments can be seen in Table 2.

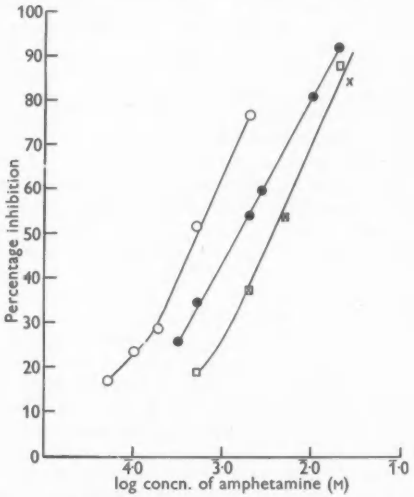

Fig. 4. Inhibition of the amine oxidase from mouse brain and kidney by (+)- and (−)-amphetamine. ×, Kidney with (−)-amphetamine; ●, kidney with (+)-amphetamine; □, brain with (−)-amphetamine; ○, brain with (+)-amphetamine. Manometer flask contents: dialysed homogenate (1 ml.) derived from 200 mg. of fresh tissue; the substrate, 5-HT, was present in each flask at an initial concentration of 10 mM; final volume, 2 ml. Inhibitor (0·2 ml.) was added to 0·2 M-sodium phosphate buffer, pH 7·4 (0·4 ml.). Gas phase, O_2; temp., 37·5°.

Experiments with 5-hydroxytryptacyamine. 5-Hydroxytryptacyamine was not oxidized when incubated with homogenates of mouse brain in concentrations up to 10 mM.

The inhibitory activity against the brain enzyme with 5-HT as substrate was relatively weak. The concentration of the guanidine necessary to inhibit the enzymic activity by 50% was 8·9 mM.

DISCUSSION

Differences in substrate specificity as well as in the relative rates of oxidation of the amines suggest the possibility that each tissue may contain a distinct enzyme for the destruction of monoamines.

In all the tissues examined tryptamine was oxidized more slowly than tyramine. Schayer, Wu, Smiley & Kobayashi (1954) concluded that in the mouse tryptamine was metabolized at a rate approximately 30% above that of tyramine. This apparent contradiction may be explained if tryptamine has a higher affinity for amine oxidase than tyramine. This is not yet known for the mouse though this explanation holds for the rat, where a similar situation exists. Rat-liver amine oxidase has a greater affinity for tryptamine than

Table 2. *Amine oxidase activity in tissues from mice treated with iproniazid*

Enzymic activity is expressed as a percentage of the normal. Injection 1 consisted of 100 mg. of iproniazid/kg. body wt. 24 hr. before killing the animals. Injection 2 consisted of two administrations of 100 mg. of iproniazid/kg. body wt. 48 and 24 hr. before killing the animals. Initial substrate concentration, 10 mM. Gas phase, O_2; temp., 37·5°. Actual q_{O_2} values (μl. of O_2 consumed/mg. of fresh tissue/hr.) are given in parentheses.

Injection	Tissue	Substrate				
		5-HT	Tryptamine	β-Phenylethylamine	Tyramine	*iso*Amylamine
1	Brain	29 (0·14)	58 (0·14)	0 (0)	19 (0·14)	0 (0)
	Kidney	23 (0·13)	11 (0·03)	25 (0·04)	20 (0·09)	—
	Liver	15 (0·10)	10 (0·10)	12 (0·11)	21 (0·57)	19 (0·17)
2	Brain	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Kidney	20 (0·11)	26 (0·07)	0 (0)	18 (0·08)	—
	Liver	16 (0·11)	10 (0·10)	14 (0·13)	20 (0·54)	21 (0·18)

for tryamine, yet when these amines are tested singly tyramine is oxidized more readily than tryptamine. When the substrates are present together, tryptamine is metabolized faster (Kobayashi & Schayer, 1955). Langemann (1955) has shown that rat-liver mitochondria destroy 5-HT more rapidly than tryptamine yet only half as rapidly as tyramine. In heart-muscle mitochondria and myofibrils, on the other hand, 5-HT and tyramine are destroyed at similar rates. Tryptamine was in all cases less rapidly oxidized than 5-HT. Thus we see evidence in rats as well as mice for the view that in a number of tissues, if not all, amine oxidase has a high affinity for the indole amines and, more especially, for 5-HT.

5-HT is optically inactive; it was therefore surprising to find that the inhibition of amine oxidase activity in brain and kidney by amphetamine was greater with the (+)- than with the (−)-isomer. The brain enzyme was more optically specific than the kidney enzyme. P. Pratesi & H. Blaschko (1957, personal communication) showed that guinea-pig liver amine oxidase is more sensitive to inhibition by (+)-amphetamine than by (−)-amphetamine.

Pletscher & Gey (1958) have recently described two stereospecific amine oxidase inhibitors derived from the α-amino acids alanine and serine. The structures of these substances are:

$$H_3C{\cdot}CH(NH{\cdot}CO{\cdot}CH_3){\cdot}CO{\cdot}NH{\cdot}NH{\cdot}CH(CH_3)_2$$

$$HO{\cdot}CH_2{\cdot}CH(NH_2){\cdot}CO{\cdot}NH{\cdot}NH{\cdot}CH(CH_3)_2.$$

In both the more active isomer *in vitro* and *in vivo* was that derived from the naturally occurring amino acid. Pletscher & Gey suggested that these differences could be partly ascribed to an optically active centre in the enzyme.

The finding that (+)-amphetamine is a considerably more potent inhibitor of mouse-brain amine oxidase than the (−)-isomer is of interest because of what is known of their effects on the central nervous systems of man and animals. Schulte, Reif, Bacher, Lawrence & Tainter (1941) studied the central stimulant activity of (+)- and (−)-amphetamine in un-narcotized rats. The (+)-isomer was three times as active on a weight basis as the (−)-isomer. Alles (1939) had previously shown by subjective observations in man that (+)-amphetamine was four times as active as (−)-amphetamine.

In their peripheral effects the isomers of amphetamine are quantitatively more alike, e.g. (+)- and (−)-amphetamine are approximately equally active in decreasing the propulsive motility of the small intestine in rats and dogs (Van Liere, Stickney, Northup & Bell, 1951). Possibly these findings are related to the observation made in the present work that brain amine oxidase displays more optical specificity than the enzyme found in a peripheral tissue (kidney).

The results obtained with mice treated with iproniazid show some differences from those obtained by Davison *et al.* (1957). Neither the brain nor the liver enzyme was fully inactivated by a single dose of iproniazid (100 mg./kg. body wt.). Approximately 20% of the activity remained in each tissue and also in kidney. A second similar dose of iproniazid did not alter the activity in liver and kidney, but the brain enzyme was fully inactivated. The rate of oxygen consumption with tryptamine as substrate was less inhibited than with other substrates in brain homogenates from mice after a single treatment with iproniazid (see Table 2). This phenomenon is probably related to that described earlier (for inhibition by cyanide) and is due to the further metabolism of the product of amine oxidase action on tryptamine.

The reason for differences between these results and Davison's is not at present known; it is possible that a strain difference is responsible. The mice used in the present studies are characterized by a high degree of hybrid vigour not possessed by albino mice used by Davison.

SUMMARY

1. 5-Hydroxytryptamine, tryptamine, tyramine, β-phenylethylamine, *iso*amylamine and benzylamine have been tested as substrates of amine oxidase in homogenates of the following mouse tissues: brain, kidney, liver, lung and spleen.

2. Mouse kidney contains a monoamine oxidase which destroys 5-hydroxytryptamine more rapidly than tyramine, tryptamine or β-phenylethylamine. This is also true of lung and spleen.

3. 5-Hydroxytryptamine was oxidized most rapidly by lung.

4. In liver homogenates 5-hydroxytryptamine was oxidized less rapidly than any other monoamine tested, yet the rate was only slightly less than that observed with lung tissue.

5. *iso*Amylamine and benzylamine, although oxidized by liver, brain and lung, were not attacked by kidney and spleen.

6. Differences in substrate specificity and relative rates of oxidation of the substrates serve to distinguish the amine oxidase from each tissue.

7. (+)-Amphetamine was seven times as potent as (−)-amphetamine as an inhibitor of brain amine oxidase; the difference was less with kidney.

8. The guanidine analogue of 5-hydroxytryptamine was synthesized. It is a weak inhibitor of monoamine oxidase. These findings are discussed in relation to the function of amine oxidase.

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Studies on the Synthesis of Serum Albumin by the Isolated Microsome Fraction from Rat Liver

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Zamecnik & Keller (1954) were the first to show that when radioactive amino acids are incubated with the microsomal fraction from rat liver, together with the soluble cytoplasm and a source of continuously regenerated adenosine triphosphate, an incorporation of amino acid into microsomal protein occurs. Since then the steps in this process of incorporation have been extensively studied; the precise nature of the radioactive microsomal proteins has remained undefined, although some progress in this direction was made by Simkin & Work (1957) by extracting the microsomes with solutions of varied ionic strength and pH. It is therefore not clear to what extent this incorporation of radioactive amino acids into substances precipitated by cold trichloroacetic acid but insoluble in hot acid represents the synthesis of a complete protein. It was in the hope that an answer to this question might be found that the present experiments were initiated.

Experiments on the perfused rat liver indicate that this organ is able to synthesize serum albumin (Miller, Bly, Watson & Bale, 1951). Moreover, it is possible to demonstrate the synthesis of serum albumin in incubated slices of either chick liver (Peters & Anfinsen, 1950) or rat liver (Campbell & Stone, 1957*a*). This demonstration depended on the use of specific antisera by which means the net synthesis of serum albumin during the incubation period could be shown. Further, if the slices were incubated in the presence of a radioactive amino acid, the serum albumin became radioactive and the degree of incorporation of radioactivity was approximately correlated with the amount of protein synthesized (Campbell & Stone, 1957*a*). Peters (1957) later showed that chick-liver microsomes contained a small amount of serum albumin which could be solubilized. It was therefore decided in the present experiments to incubate rat-liver microsomes in the presence of radioactive amino acids under the conditions described by Zamecnik & Keller (1954) and to determine whether the amino acids were incorporated into the serum albumin which could be released from the microsomes. It was assumed that it would not be practicable to demonstrate the net synthesis of serum albumin in such preparations.

The major difficulty in conducting these experiments has been to demonstrate unequivocally that the isolated radioactive protein has all the characteristics of serum albumin. As will be shown, immunological, electrophoretic and solubility criteria have been applied in an attempt to demonstrate this. The use of a wide variety of criteria is particularly important for serum albumin since this protein retains its immunological characteristics even though it has been modified, e.g. by acetylation (Marrack & Orlans, 1954), or split into smaller fragments (Lapresle, 1955; Porter, 1957).

Since the amount of albumin which can be released from the liver microsomes is very small, it has been necessary to take advantage of every procedure which would ensure the isolation of albumin with the highest possible radioactivity. It has previously been shown (Hultin & Decken, 1957; Decken & Hultin, 1958; Campbell & Greengard, 1959) that the rat-liver microsome fraction derived from rats after partial hepatectomy is much more

active with respect to incorporation of amino acids than is a similar fraction from normal rats. Most of the experiments have therefore been conducted with microsomes from regenerating liver.

The present experiments show that it is possible to isolate from the microsome fraction of rat liver that has been incubated with radioactive amino acids a protein which is radioactive and which retains all the properties of serum albumin for which it has been tested. The results are consistent with the hypothesis that the isolated microsomal fraction of rat liver is able to synthesize a specific protein. Preliminary reports of some of the results of these experiments have previously appeared (Campbell, Greengard & Kernot, 1958; Campbell & Greengard, 1958; Campbell, 1958*b*).

While these experiments were in progress similar studies on two other biological systems have also been reported. Thus Schweet, Lamfrom & Allen (1958) have demonstrated the incorporation of radioactive amino acids into haemoglobin by the isolated microsome fraction from rabbit reticulocytes. Bates, Craddock & Simpson (1958) have demonstrated the incorporation of radioactive amino acids into cytochrome *c* during the incubation of rat-liver mitochondria. Recently a net synthesis of this protein by mitochondria isolated from calf heart has been shown (Bates & Simpson, 1959).

MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (dipotassium salt; ATP) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Solutions of this substance were brought to pH 7·3 (indicator papers) with KOH before use. The barium–silver salt of phosphoenolpyruvic acid was prepared by the method of Ohlmeyer (1951). Solutions of the free acid were prepared shortly before use and adjusted to pH 7·3 (indicator papers) with KOH.

Radioactive amino acids. Uniformly labelled L-[^{14}C]-leucine (100 μc, 2·29 mg.), L-[^{14}C]lysine (100 μc, 1·13 mg.), L-[^{14}C]valine (100 μc, 1·46 mg.) and an acid hydrolysate of algal [^{14}C]protein (100 μc, 2·7 mg.) were obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Rats were male Wistar albinos highly inbred at this Institute. Partial hepatectomy was performed by the method of Higgins & Anderson (1931).

Preparation of rat-serum albumin. This was prepared from rat serum by a slight modification of the method described for human albumin by Debro, Tarver & Korner (1957). An equal volume of 10% (w/v) trichloroacetic acid was added to the serum, the supernatant after centrifuging was rejected and the precipitate was washed with 5% (w/v) trichloroacetic acid. The precipitate was then suspended in 1% (w/v) trichloroacetic acid in aq. 96% ethanol in a volume equivalent to three times the original volume of serum. After centrifuging, the extract was dialysed against distilled water and freeze-dried.

Chick-serum albumin. This was the sample the preparation of which was previously described (Campbell, 1955).

Antisera against rat- and chick-serum albumin. Rabbits were injected with alum-precipitated albumin as described by Campbell (1955). The amount of antibody in the rabbit serum was estimated by serial dilution to be about 10 mg./ml. of serum.

Immunological examination. The purified rat-serum albumin and the extracts from the liver microsomes were tested for antigenic heterogeneity by precipitation in agar either in tubes as described by Oudin (1948) or in plates as described by Ouchterlony (1953). The immunoelectrophoretic method of Kohn (1958) employing cellulose acetate strips was also used.

Experiments in the whole animal

Injection. In the studies on normal liver, a rat (150 g. body wt.) was injected intravenously (femoral vein) with 1 ml. of 0·9% NaCl containing 20 μc of algal [^{14}C]protein hydrolysate. In the studies on regenerating liver, two rats (150 g. body wt.) were subjected to partial hepatectomy and after 48 hr. each rat received by intravenous injection 0·5 ml. of 0·9% NaCl containing 10 μc of algal [^{14}C]protein hydrolysate.

Fractionation of liver. Both the normal and operated rats were killed 20 min. after the injection. The liver was rapidly removed and homogenized in 2 g. samples in an all-glass Potter homogenizer with 10 ml. of 0·25 M-sucrose. The tissue suspension was filtered through a double layer of gauze and the nuclear, mitochondrial and microsomal fractions were obtained according to the method of Schneider & Hogeboom (1950). The fractions were stored at −15° until required.

Experiments involving the incubation of isolated microsomes

Preparation of microsomes. Regenerating liver was taken from rats 44–48 hr. after partial hepatectomy. Microsomal preparations were made by a method which followed closely that described by Zamecnik & Keller (1954). The tissue was homogenized in 2·5 times its weight of a medium containing phosphate buffer [0·01 M-$MgCl_2$, 0·02 M-potassium phosphate buffer (pH 7·8), 0·03 M-$KHCO_3$, 0·025 M-KCl and 0·35 M-sucrose]. The tissue suspension was centrifuged at 12 000 *g* for 5 min. The supernatant from this centrifuging was then centrifuged in a model L Spinco preparative centrifuge (no. 40 rotor) for 50 min. at 105 000 *g*. Some of the clear supernatant was siphoned off with a syringe, leaving behind the fat-rich supernatant. The remainder of the supernatant was decanted and rejected. The pellet was suspended, in 0·4 ml. of medium/g. of original liver, by gentle homogenization in a small all-glass Potter homogenizer. Care was taken to keep all preparations ice-cold during these procedures.

Method of incubation. In the small-scale experiments, the incubation mixture contained 0·4 ml. of the microsome suspension, 0·3 ml. of cell sap (i.e. clear supernatant after centrifuging at 105 000 *g*), 2 μmoles of ATP, labelled amino acid and 10 μmoles of phosphoenolpyruvic acid, in a total volume of 1 ml. In the large-scale experiments the same proportion of reagents was used, but all volumes were multiplied by a factor of 5 and the incubation took place in 50 ml. Erlenmeyer flasks. After incubation for 50 min. under a continuous stream of N_2+CO_2 (95:5) at 37°, the microsomes were isolated from the incubation mixture by further centrifuging at 105 000 *g* for 50 min.

Isolation of proteins

Mixed microsomal or cell-sap proteins. The protein was precipitated by the addition of an equal volume of 10% (w/v) trichloroacetic acid. The protein was then treated with hot trichloroacetic acid and organic solvents according to a modified Schneider procedure (Zamecnik, Loftfield, Stephenson & Steele, 1951).

Serum albumin. When serum albumin was to be isolated from soluble fractions the isolation procedures could be applied directly. However, if the serum albumin present in the microsome fractions was being studied, it was necessary first to disintegrate the particles as follows. The particles from approximately 1 g. of liver were suspended in 2 ml. of 0·9% NaCl by homogenizing in a Potter homogenizer and were then treated for 2 min. with ultrasonics. For this purpose an ultrasonic drill (Mullard Ltd., London; type E7680, 16–24 kcyc./sec.) was used with a stainless-steel probe which had the following dimensions: narrow end 0·25 in. external diameter, 2·5 in. long; wide end 0·75 in. external diameter, 2·5 in. long. The particulate suspension was placed in a glass tube (0·5 in. × 2·5 in.) which was held in an ice bath. The suspension was then frozen and stored at −15° overnight. Before extraction of albumin the suspension was thawed and centrifuged at 105 000 *g* for 50 min. to remove the microsomal debris.

Precipitation of albumin by antiserum. The albumin in the clear extracts was isolated as an antibody–antigen complex by the addition of a specific antiserum as described by Campbell & Stone (1957*a*, *b*). Before the rat-albumin antiserum was added the tissue extracts were 'cleared' by the addition of chick albumin, which was removed by the addition of chick-albumin antiserum as previously described.

Isolation of albumin involving electrophoresis. To the tissue extracts was added 2 mg. of rat albumin in 0·9% NaCl. The total proteins were then precipitated by the addition of an equal volume of 10% (w/v) trichloroacetic acid, washed with 5% (w/v) trichloroacetic acid (1 ml.) and the precipitate was treated with 2 ml. of 1% (w/v) trichloroacetic acid in aq. 96% ethanol. This suspension was centrifuged and the residue again extracted with 1% (w/v) trichloroacetic acid in aq. 96% ethanol (1 ml.). The supernatants were combined and dialysed against distilled water. To ensure that the final volume of the residue should be as small as possible, the minimum length of narrow-bore dialysis tubing was used. The precipitate that formed on dialysis was then removed by centrifuging and the clear supernatant (about 5 ml.) was freeze-dried from a 25 ml. round-bottom flask with a B24 standard ground-glass joint. The male joint on the freeze-drier was covered with a piece of lens tissue to collect any of the dried protein that escaped from the flask.

In order to isolate the albumin from the freeze-dried protein, the electrophoresis method of Kohn (1957) was adopted. The protein (1·0–1·5 mg.) was dissolved in 20 μl. of veronal buffer (1·1%, w/v, sodium barbitone and 0·15%, diethylbarbituric acid, pH 8·6; *I* 0·05) containing a few crystals of bromophenol blue and was applied with a micropipette to a strip of cellulose acetate paper (20 cm. × 5 cm.; obtainable from Oxo Ltd., Oxoid Division, Thames House, London, E.C. 4) in a straight line approximately 6 cm. from the cathode end. A sample (0·5 mg.) of a rat-serum albumin preparation obtained by fractionation of rat serum with Na_2SO_4 was run on a parallel marker strip. A current of 2·6 mA (150 v) was applied for 5–7 hr. The albumin band migrated at about 1 cm./hr. It was usually possible after this time to distinguish clearly the position of the albumin band by the light blue of the complex between albumin and bromophenol blue. If this was not so, the position of the albumin band on the experimental strip was estimated from the relative position on the marker strip. After the completion of electrophoresis the marker strip was dried in an oven at 90° for 5 min. and was then stained with 0·1% (w/v) azocarmine in a solution containing 0·45 M-acetic acid and 0·045 M-sodium acetate. After washing the excess of dye from the marker strip with 10% (w/v) acetic acid, the position of the albumin band could again be checked. Having by this means determined the relative position of the albumin on the experimental strip, a section of the appropriate width was cut out and the paper placed in 5 ml. of 0·9% saline. The remainder of the strip was then stained with azocarmine to check the location of any other protein components.

The strip of cellulose acetate paper containing the albumin was then finely cut with scissors and finally disintegrated in a small Potter-type homogenizer. The suspension was cleared by centrifuging.

Measurement of radioactivity

Mixed proteins. Proteins precipitated with trichloroacetic acid were compressed on to 0·28 cm.^{2} Perspex disks and counted at infinite thickness with a thin mica end-window (2 mg./cm.^{2}) Geiger–Müller counter. For details of the counting procedure and accuracy, see Campbell & Greengard (1959).

Albumin. The method was very similar to that previously described by Campbell & Stone (1957*a*). The albumin-antibody precipitates were dissolved in 0·4 ml. of aq. 5 N-NH_3 solution and the solution was transferred to 2 cm.^{2} polythene disks and dried in a vacuum desiccator. When dry, the disks could be counted at infinite thinness and the amount of protein on the disks determined afterwards by dissolving the protein in 0·1 N-NaOH and measuring the extinction of the solution at 280 mμ. The disks were either counted under the mica end-window counter, as in (*a*) above, or in a Tracerlab SC-16 windowless flow counter.

Homogeneity of rat-serum albumin preparations

The rat-serum albumin used for the preparation of antiserum was prepared by the ethanol–trichloroacetic acid method of Debro *et al.* (1957). For the evaluation of the metabolic experiments it was essential to determine the homogeneity of the albumin preparations. This was done as follows.

Electrophoresis. Compared with either human- or rabbit-serum proteins, it is difficult to separate the α_1-globulin and albumin components of rat-serum proteins by electrophoresis on paper. However, as shown by Campbell, Kernot & Roitt (1959), this separation may be achieved by the use of cellulose acetate paper, following the method of Kohn (1957). With this method it could be shown that the rat-serum albumin preparation was not contaminated by any other known serum protein.

Immunological methods. When the serum albumin and its antiserum were allowed to diffuse in agar gel in tubes, following the method of Oudin (1948), no evidence of heterogeneity could be detected. Concentrations of antigen

ranging from 10·0 to 0·1 mg./ml. were used. The agar-diffusion method of Ouchterlony (1953), in which diffusion takes place in Petri dishes, was also used. The antiserum to rat-serum albumin was placed in the centre well and rat albumin and whole rat serum were placed in alternate outer wells. Only one precipitation line appeared opposite each well and all the lines merged completely. This indicated that the antiserum reacted only with the albumin component of the whole serum. When the specificity of the antiserum to chick-serum albumin was studied by the Ouchterlony technique no evidence of any cross-reaction of the antiserum with rat albumin could be found.

Preparative electrophoresis on cellulose acetate paper

Resolution of serum proteins. As was previously found (Campbell *et al.* 1959), the α_1-globulin contained in the crude rat-serum albumin preparation which was used for the marker strip was always well separated from the albumin band. Care was taken not to cut too wide a strip from the albumin band on the experimental paper. In fact, when the remaining paper was stained with azo-carmine some albumin was invariably found on either side of the strip which had been removed.

Amount of protein treated. The major disadvantage of the use of cellulose acetate paper is the low absorptive capacity of the paper for water. Thus with the size of paper used in the present experiments not more than 20 μl. of solution could be absorbed on the strip, which meant that only a proportion of the freeze-dried protein could be transferred to the paper. In practice this usually amounted to 0·5–1·0 mg., which was the maximum amount of protein that could be fractionated satisfactorily.

Recovery of protein from the paper. A known amount of rat-serum albumin was placed on the paper and, after application of the current for 7 hr., the albumin band was cut out. As explained above, a small amount of albumin was left on the paper around the portion which was removed. The paper was treated as described above and the amount of albumin determined by use of an antiserum (Campbell & Stone, 1957*a*). The amount of albumin in the standard solution applied to the paper was similarly determined. It was thus shown that approximately 60% of the albumin placed on the paper was recovered.

Release of albumin from microsomes by ultrasonic disintegration

In order to determine the extent of the release of serum albumin from liver microsomes the microsomal fraction was isolated from normal rat liver. Microsome fractions from 1 g. of liver were dispersed in a small Potter homogenizer in 2 ml. of 0·9% NaCl. In one case the suspension was then cleared by ultracentrifuging and in the other the suspension was subjected to ultrasonic treatment and then cleared by ultracentrifuging. The amount of rat-serum albumin in the two supernatants was then determined after treating each with chick-serum albumin and its antiserum. The results of the estimations showed that the rat-serum albumin content of the supernatants was increased from 103 to 147 μg. of albumin/ml. by the ultrasonic treatment.

RESULTS

Incorporation of amino acids into microsomal proteins in vivo

In order to interpret the results of the studies with isolated microsomes it was considered essential to study the incorporation of amino acids under conditions in which protein synthesis was known to occur, i.e. in the whole animal. Accordingly both normal rats and animals which had been subjected to partial hepatectomy were injected with a mixture of radioactive amino acids. The rats were killed at a time after injection when it was expected that the radioactivity of the total protein of the microsome fraction would exceed that of the other subcellular fractions.

The specific activity of the protein of the various subcellular fractions is given in Table 1. This shows that for both types of liver the microsomal protein

Table 1. *Radioactivity of the protein of the subcellular fractions of rat liver 20 min. after the intravenous injection of* [^{14}C]*amino acids*

Radioactivity is expressed as counts/min. at infinite thickness/0·28 cm.^{2} with the mica end-window counter. The results obtained with the two types of liver are not comparable.

Fraction	Normal liver	Regenerating liver
Nuclear	102	72
Mitochondria	119	87
Microsome	413	177
Cell sap	78	65

Table 2. *Radioactivity of the rat-serum albumin present in the supernatants of the disintegrated microsomes obtained from the liver of rats 20 min. after injection of* [^{14}C]*amino acids*

The microsome pellets were homogenized, treated with ultrasonics, frozen and the debris was removed. Chick-serum albumin (60 μg.) was added to the supernatants and was then removed by the addition of antiserum. Rat albumin was then precipitated from the supernatants by the addition of specific antiserum. The radioactivity of the antibody precipitates was determined at infinite thinness with the windowless flow counter. Specific activity is expressed as counts/min./mg. of albumin. The results obtained with the two types of liver are not comparable.

	Normal liver		Regenerating liver	
	Counts	Specific activity	Counts	Specific activity
Chick albumin	216	3 600	128	2130
Rat albumin	599	13 450	382	3638

had a higher radioactivity than that of the other fractions.

Table 2 shows the radioactivity of the rat-serum albumin in the supernatants of the disintegrated microsomes. The amount of serum albumin released from the microsome pellet obtained from approximately 1 g. of liver was 78 μg. for normal liver and 22 μg. for regenerating liver. In order to check that the radioactivity of the precipitate of rat albumin was not due to the non-specific adsorption of other radioactive substances, chick albumin was first added to the extracts and was then removed by the addition of an antiserum to chick albumin (Campbell & Stone, 1957*a*, *b*). It will be seen that the chick albumin precipitates had a considerable activity but that this was less than that of the rat-albumin precipitates. In another experiment the treatment with chick albumin was carried out twice, the activity of the second precipitate being only one-third of that of the first.

In Table 3 the results of the determination of the radioactivity of the microsomal albumin before and after its isolation by the trichloroacetic acid–ethanol procedure are given. The fact that the isolation procedure increases the specific activity of the serum albumin suggests that the albumin-antiserum precipitates obtained from the original extract were contaminated with substances having an absorption at 280 mμ with a lower specific radioactivity than that of the rat-serum albumin.

From Table 4 it will be seen that the specific radioactivity of the rat-serum albumin from normal liver is reduced as a result of electrophoresis but that from regenerating liver is not significantly affected. Evidence that the radioactivity remained associated with the albumin even after electrophoresis was obtained by combined radioautography on an X-ray film (Kodirex, Kodak Ltd., London) and immunoelectrophoresis (Kohn, 1958). These methods showed that whereas much of the radioactivity was present in the albumin fraction there was a radioactive band at the point of application of the protein to the paper. This was probably due to denatured albumin.

Table 5 shows a comparison of the radioactivity of the albumin extracted from the microsome fraction, before purification by the trichloroacetic acid–ethanol procedure, with that of the insoluble microsomal proteins. To calculate these results use was made of the factor previously determined (Campbell & Stone, 1957*a*) for converting counts/min./mg. of protein at infinite thinness into counts/min. at infinite thickness/cm.^{2}. It will be seen that the serum albumin is much more radioactive than is the insoluble protein although the difference is much smaller for regenerating liver than for normal liver. In regenerating liver the synthesis of tissue protein might be expected to be relatively faster than in normal liver. Severina (1956) found that whereas the rate of synthesis of serum albumin by slices of regenerating chick liver was depressed below normal 24 hr. after operation it rose above normal after 2 days.

Table 3. *Radioactivity of rat-serum albumin after isolation by trichloroacetic acid–ethanol from supernatants of the disintegrated microsomes from regenerating liver labelled* in vivo

Carrier rat-serum albumin (2 mg.) was added to the supernatant containing about 1·2 mg. of albumin and the radioactivity of the rat-serum albumin was determined on a sample as described in Table 2. The rat-serum albumin was then isolated by the trichloroacetic acid–ethanol procedure (see Methods section), and the activity of this purified albumin was again determined by the use of the antiserum. Radioactivity is expressed as counts/min./mg. of albumin.

	Before isolation	After isolation
Chick albumin	503	—
Rat albumin	1084	1560

Table 4. *Effect of electrophoresis on the radioactivity of rat-serum albumin isolated from the supernatants of disintegrated microsomes from normal and regenerating liver labelled* in vivo

The rat-serum albumin which had been purified by the trichloroacetic acid–ethanol procedure (see Tables 2 and 3) was subjected to electrophoresis on cellulose acetate paper (see Methods section). The albumin was removed from the paper and precipitated by the addition of antiserum. The radioactivity of the precipitate was determined with the windowless flow counter. Results are expressed as counts/min./mg. of albumin.

	Normal liver	Regenerating liver
Before electrophoresis	1375	1560
After electrophoresis	1083	1531

Table 5. *Comparison of radioactivity of albumin isolated from the microsomes and insoluble microsomal protein after intravenous injection of* [^{14}C]*amino acids*

Results are expressed as counts/min. at infinite thickness/cm.^{2} with the mica end-window counter.

	Insoluble protein	Albumin	Ratio: Albumin / Insoluble protein
Normal liver	970	20 900	22
Regenerating liver	730	5 400	8

Studies on isolated microsomes

In the initial experiments in which the microsome fraction from regenerating rat liver was incubated with radioactive amino acids, a source to regenerate ATP and the cell sap, the methods used for the characterization of the serum albumin were entirely immunological. The results of a typical experiment are shown in Tables 6 and 7. Table 6 shows that the presence of phosphoenolpyruvate had a marked effect on the incorporation of radioactive amino acids both for the microsomal protein and the cell-sap protein. From Table 7 it will be seen that the presence of phosphoenolpyruvate greatly enhanced the specific radioactivity of the microsomal rat albumin. It seems probable that the activity of the latter was not due merely to contamination with other radioactive substances since the chick-albumin precipitates had a much lower radioactivity than that of the rat albumin. However, with the cell-sap proteins there was no significant difference in the activity of the rat albumin in the presence and absence of phosphoenolpyruvate. Moreover, the radioactivity of the chick-albumin precipitates was so high that the apparent activity of the rat-albumin precipitates could well have been due to contamination with radioactive substances other than rat albumin. The activity of the second chick-albumin precipitate was in every case less than that of the first. However, with microsomal albumin in the presence of phosphoenolpyruvate the difference was not significant. In a similar experiment a further treatment with chick albumin was carried out after the precipitation of the rat albumin. Here again the chick-albumin precipitate had a similar activity to that of the other chick-albumin precipitates. Approximately 30 μg. of rat albumin was obtained from the microsome pellet equivalent to 1 g. of rat liver. Since such a pellet contains about 5 mg. of protein the albumin represents 0·5–1·0 % of the total microsomal protein.

A similar experiment to that just described was done with microsomes isolated from normal liver, except that [^{14}C]leucine and [^{14}C]valine were used. The insoluble protein from the microsomes had a radioactivity of 27 and 562 counts/min. at infinite thickness/0·28 cm.^{2} respectively when the incubation was carried out in the absence and presence of phosphoenolpyruvate. The results of the determination on the radioactivity of the extracted albumin are shown in Table 8. The specific activity of the rat-albumin precipitate in the presence of phosphoenolpyruvate was much greater than in its

Table 6. *Incorporation of* [^{14}C]*leucine and* [^{14}C]*lysine into microsomal and cell-sap proteins in isolated preparations from regenerating liver*

Microsomes and cell sap (in quantities given in the Methods section) were incubated under $N_2 + CO_2$ (95:5) for 50 min. at 37° with 2 μmoles of ATP, and 2 μc each of [^{14}C]leucine and [^{14}C]lysine with and without 10 μmoles of phosphoenolpyruvate (PEP) in a total volume of 1 ml. The microsomes were isolated after incubation by centrifuging and were ultrasonically disintegrated. The rat albumin was removed from the solubilized proteins by the addition of antiserum. The microsomal protein refers to the insoluble proteins together with the non-albumin soluble protein. The soluble fraction of the incubation mixture containing the cell sap was freed of rat albumin by the addition of antiserum. Cell-sap protein therefore refers to the soluble proteins of this fraction other than albumin. Radioactivity is expressed as counts/min. at infinite thickness/0·28 cm.^{2} in the mica end-window counter.

	Microsomal protein		Cell-sap protein	
	Control	Plus PEP	Control	Plus PEP
Radioactivity	69	738	9	189

Table 7. *Radioactivity of rat-serum albumin isolated from the microsomes and cell-sap protein of regenerating liver after incubation with* [^{14}C]*lysine and* [^{14}C]*leucine*

The supernatants obtained after treatment of the microsomal pellet with ultrasonics were 'cleared' twice by the addition of chick-serum albumin (60 μg.) and its subsequent removal with antiserum. The soluble fraction from the incubation mixture was similarly treated. Finally the rat-serum albumin in each fraction was precipitated by the addition of antiserum to rat albumin. Specific radioactivity is expressed as counts/min./mg. of albumin as determined in the mica end-window counter. PEP, Phosphoenolpyruvate.

	Soluble fraction from disintegrated microsomes				Soluble fraction from incubation mixture			
	Control		Plus PEP		Control		Plus PEP	
	Counts/ min.	Specific activity	Counts/ min.	Specific activity	Counts/ min.	Specific activity	Counts/ min.	Specific activity
Chick albumin 1	17	284	29	484	152	2530	178	2970
Chick albumin 2	4	67	28	468	72	1200	127	2120
Rat albumin	9	50	227	7100	145	805	153	845

Table 8. *Radioactivity of rat-serum albumin isolated from normal rat-liver microsomes incubated with* [^{14}C]*leucine and* [^{14}C]*valine*

The incubation procedure was as described under Table 6 except that all quantities were increased by a factor of 5, as described in the Methods section. To each flask containing 5 ml. of the incubation mixture 5 μc each of [^{14}C]leucine and [^{14}C]valine was added. Rat albumin was obtained from the microsome pellet after incubation by treatment with ultrasonics. Results are expressed as counts/min./mg. of albumin at infinite thinness as determined in the windowless flow counter. PEP, Phosphoenolpyruvate.

	Control		Plus PEP	
	Counts/min.	Specific activity	Counts/min.	Specific activity
Chick albumin 1	14	230	188	3120
Chick albumin 2	7	115	103	1710
Rat albumin	7	500	164	5870

Table 9. *Effect of various isolation procedures on the radioactivity of albumin isolated from microsomes obtained from regenerating rat liver, incubated with* [^{14}C]*leucine and* [^{14}C]*valine*

The procedure for the incubation was as described in the Methods section under large-scale experiments. Each incubation flask containing 5 ml. of the incubation mixture contained 7·5 μc each of [^{14}C]valine and [^{14}C]leucine, together with phosphoenolpyruvate, ATP and cell sap. Controls without phosphoenolpyruvate were not carried out.

(*a*) Procedures applied directly to the supernatant from disintegrated microsomes. For details see Table 7.

	Counts/min.	Specific radioactivity
Chick albumin 1	51	850
Chick albumin 2	29	500
Rat albumin	67	5350

(*b*) Carrier rat-serum albumin (2 mg.) was added to the supernatant from the disintegrated microsomes (containing approx. 200 μg. of albumin) and the albumin re-extracted by the trichloroacetic acid–ethanol procedure. Some of this albumin was then subjected to electrophoresis. The results are the average of two determinations. In another determination the extracts were 'cleared' with chick albumin before addition of rat-albumin antiserum. The chick-albumin precipitates were not significantly radioactive. Radioactivity was determined with a mica end-window counter. Results are expressed as specific activities of albumin (counts/min./mg.).

	Before electrophoresis	After electrophoresis	Change (%)
Specific activity	325	238	26
Specific activity after clearing	410	273	33

absence. However, compared with regenerating-liver preparations the contamination of the chick albumin is much greater with normal liver microsomes. This may be due to the fact that the amount of radioactive amino acid used for both preparations is similar but that the incorporation of radioactivity is much greater with regenerating-liver preparations.

Several experiments were carried out with larger amounts of microsomes from regenerating liver in order that sufficient radioactive albumin might be obtained to check the effect on its radioactivity of purification by the trichloroacetic acid–ethanol procedure and electrophoresis. The results of a typical experiment are shown in Table 9. In this experiment the radioactivity of the insoluble protein from the microsomes was 2742 counts/min. at infinite thickness/0·28 cm.^{2}. As seen in Table 9 (*a*), the specific activity of the microsomal rat albumin was very much higher than that of the chick-albumin precipitates, which confirms the previous experiments. It may be observed, Table 9 (*b*), that the activity of the rat albumin was reduced by electrophoresis. The effect of 'clearing' the extracted protein both before and after electrophoresis was to increase slightly the specific activity of the rat albumin.

In another experiment carried out under exactly the same conditions the results obtained were very close to those just described. The opportunity was taken to determine the effect on the specific radioactivity of the microsomal albumin of the trichloroacetic acid–ethanol step. The activity dropped from 423 to 319 counts/min./mg. of albumin. This is in contrast with the effect of this procedure on the microsomal albumin labelled *in vivo*. In two respects the results in this experiment did differ from those of the previous one. First, the precipitate of the chick albumin used to 'clear' the microsomal albumin isolated by the trichloroacetic acid–ethanol procedure was significantly radioactive. However, the second chick-albumin precipitate was inactive and in no case was there any activity in such precipitates when the rat albumin had first been subjected to electrophoresis. Secondly, the reduction in the specific radioactivity of the rat albumin on electrophoresis was greater than previously, being 46 % instead of 26 %. The effect of clearing with chick albumin on the specific radioactivity of the rat albumin was to reduce the activity of the protein before electrophoresis but not to alter that of the protein after electrophoresis.

Table 10. *Comparison of the radioactivity of microsomal albumin and insoluble microsomal protein after incubation of microsomes from both normal liver and regenerating liver with* [^{14}C]*leucine and* [^{14}C]*valine*

Results are expressed as counts/min. at infinite thickness/cm.^{2} with the mica end-window counter and were calculated from the data in Tables 8 and 9.

	Insoluble protein	Albumin	Ratio: Albumin / Insoluble protein
Normal liver	2220	9 170	4·1
Regenerating liver	8800	25 700	2·9

A radioautograph of the cellulose acetate strip used for the electrophoresis of the rat-serum albumin showed that, apart from the radioactivity due to the denatured protein at the point of application, the activity was concentrated around the area from which the albumin had been extracted.

Just as the radioactivity of the microsomal albumin could be compared with that of the insoluble microsomal proteins after intravenous injection of radioactive amino acids (see Table 5), so a similar calculation can be made for the experiments *in vitro*. The results of such a calculation are shown in Table 10.

In both these and the earlier experiments the rat albumin in the medium after incubation of the microsomes appeared to have some radioactivity when precipitated by addition of rat-albumin antiserum. However, after addition of carrier rat albumin to the extracts and isolation by the trichloroacetic acid–ethanol method the activity was always low and sometimes the rat albumin was inactive. Electrophoresis removed any remaining activity in these samples of rat albumin. Thus in no case was rat albumin which retained its radioactivity after electrophoresis isolated from the incubation mixture.

DISCUSSION

It must be accepted that the demonstration of a net synthesis is the best criterion of the synthesis of a complex substance such as serum albumin under conditions *in vitro*. In liver slices such a net synthesis of serum albumin during the period of incubation has been demonstrated but at least two considerations made it unlikely that such a criterion could be successfully applied to a cell-free system. First, studies with radioactive amino acids have indicated that, compared with slices, the protein-synthetic activity of isolated liver microsomes is comparatively low and rapidly declines on incubation at 37°, and, secondly, incorporation of radioactive amino acids, and hence protein synthesis, depends on the incubation of the liver microsomes in the presence of cell sap which contains serum albumin. Attempts to reduce the amount of serum albumin in the incubation mixture, by substituting whole cell sap by pH 5·0 fraction (Keller & Zamecnik, 1956), failed because precipitation at pH 5·0 did not effect any significant degree of fractionation as between cell-sap proteins and serum albumin. Because of these difficulties it has been necessary to use the incorporation of radioactive amino acids into serum albumin as the criterion of protein synthesis. Although it follows from definition that proteins are not synthesized in the absence of incorporation of amino acids, the mere demonstration of such incorporation cannot be taken as unequivocal evidence of protein synthesis *de novo*. The reasons for this conclusion have already been discussed at length (e.g. Campbell, 1958*a*), so that it will suffice here to confine the discussion to matters particularly relevant to the present problem.

Whereas for many years the microsome fraction remained only as a cytoplasmic concept a much better understanding of the morphological structure of this fraction, at least for rat liver, has arisen from the work of Palade & Siekevitz (1956), who have shown that the rat-liver microsome fraction corresponds mainly to the rough endoplasmic reticulum of the cytoplasm. In view of this it was not surprising that Peters (1957) should find some serum albumin in the soluble protein obtained from the chick-liver microsomes by treatment with deoxycholate. Peters showed that when chick-liver slices were incubated under conditions in which there was evidence of protein synthesis albumin was released from the microsomes.

The results of the present experiments with isolated microsomes, in which the rat-serum albumin was initially only characterized by immunological methods, show that the incorporation of radioactive amino acids was dependent on the presence of a source of continuously regenerated adenosine triphosphate (Table 7). It was necessary to show that this was so because of the amino acid–protein interactions demonstrated by Cornwell & Luck (1958). The use of chick albumin, and its subsequent removal by its antiserum, to check the specificity of precipitation of rat-serum albumin by its antiserum has been studied previously (Campbell & Stone, 1957*a*, *b*). A similar method has also

been used by Peters (1957). With microsomal albumin the radioactivity of the chick-albumin precipitates was small compared with that of the rat-albumin precipitates and it is unlikely that the radioactivity of the rat-albumin precipitates was due to the co-precipitation of other radioactive substances. However, the fact remains that the chick-albumin precipitates were radioactive and remained so irrespective of the number of times the process was repeated. The immunological studies appeared to exclude the possibility that the radioactivity of the chick-albumin precipitates was due to a cross-reaction between the chick antiserum and the rat albumin. To determine whether the co-precipitated substances were nucleic acid in nature, the absorption of the dissolved precipitates was determined at both 260 and 280 mμ. However, no evidence of any nucleotides was found so that the reason for the persistent radioactivity of the chick-albumin precipitates remains obscure.

Although these results on microsomal albumin were encouraging it was felt that dependence on a single criterion for the characterization of the radioactive albumin was unsatisfactory. Moreover, Lapresle (1955) and Porter (1957) have shown that fragments of the albumin molecule retain at least some of the immunological properties of the native albumin. The method of Debro *et al.* (1957), in which serum albumin is isolated from an ethanolic extract of a trichloroacetic acid precipitate, has proved to be an extremely useful procedure for the isolation of albumin. When this method is applied to serum proteins there is much evidence that the protein that is obtained consists almost entirely of albumin. However, when the method is applied to the cell-sap proteins subsequent electrophoresis indicates that proteins other than albumin are usually present, although with microsomal extracts the method appears to yield uncontaminated albumin. Therefore, at least for microsomal protein, the specificity of the trichloroacetic acid–ethanol method is such that it alone provides a good test of the purity of the extracted albumin, so that the retention of radioactivity in the albumin isolated from the incubated microsomes by this method amply confirmed the preliminary results.

The final criterion applied to test the purity of the microsomal albumin was electrophoresis. The use of cellulose acetate for the preparative electrophoresis of submilligram amounts of protein has been fully justified. In the experiments *in vitro* the radioactivity of the albumin was reduced as a result of electrophoresis. The percentage reduction in activity (26 and 46 % respectively in two experiments) was greater than that found with albumin in which the incorporation of radioactive amino acids had taken place *in vivo*, but there could be no doubt that the albumin remained significantly radioactive after electrophoresis.

When the rat albumin which had been purified by electrophoresis was treated with chick albumin and its specific antiserum the precipitate formed was without significant radioactivity. This is good evidence that the radioactivity of the microsomal rat albumin was not due to contamination and also demonstrates the value of the electrophoresis procedure.

The comparison which has been made between the results obtained *in vivo* and *in vitro* is a guide to the extent to which the isolated microsomes retain their activity shown *in vivo*. The results *in vivo* both for normal liver and regenerating liver show that the microsomal albumin is much more radioactive than the insoluble or structural protein of the microsomes (Table 5). The difference between the two fractions was much less in regenerating liver than in normal liver, presumably due to the high growth rate of the former relative to the latter. The results *in vitro* (Table 10) do not show much difference between the two types of liver, but again the radioactivity of the microsomal albumin was greater than that of the structural proteins. The difference in the activity of the two fractions was never as great *in vitro* as *in vivo*. This suggests a relatively greater loss in the ability of the isolated microsomes to synthesize albumin as opposed to structural protein.

The experiments with isolated liver microsomes may be said to support the hypothesis that such a tissue preparation is able to synthesize serum albumin. It is, however, difficult to be more precise than this for it cannot as yet be certain that such a preparation is able to assemble all the amino acids required for the synthesis of a complete molecule of serum albumin. The possibility that only a step in this complex process is being studied is a very real one (see, for example, Loftfield, 1958), and further experiments must be designed to determine whether this is so. In this connexion it is a pity that the terminal amino acids in rat albumin have not so far been determined as they have for the serum albumin of many other species (Peters, Logan & Sanford, 1958).

These experiments with the isolated microsome fraction do not in any way exclude the possibility that other organelles of the liver cell are able to synthesize serum albumin. Indeed the demonstration that mitochondria from chick liver (Peters, 1957) and rat liver (Campbell & Greengard, 1958) contain serum albumin makes this protein a most suitable choice for such studies. The experiments of Bates *et al.* (1958) on the synthesis of cytochrome *c* by rat-liver mitochondria are of particular significance in this connexion. Experiments are being carried out to determine the relative contribution

of the various organelles to the synthesis of serum albumin by the liver cell *in vivo*.

It remains to discuss the results of the studies on the incorporation of amino acids into the serum albumin in the supernatant obtained from the medium in which the microsomes were incubated. In the initial experiments, although the rat-albumin precipitates were radioactive there was no evidence of an energy-dependent incorporation (Table 7). The radioactivity of the chick-albumin precipitates was such that the activity of the rat albumin could have been due to coprecipitation of other substances. When the albumin from the incubation mixture was purified by the trichloroacetic acid–ethanol procedure and subjected to electrophoresis no significant radioactivity remained. This again demonstrates the value of these procedures for the removal of contaminating substances. The soluble fraction of the incubation mixture contains approximately twice as much albumin at the end of incubation as can be recovered from the microsomes. Thus it is possible that the failure to detect radioactive albumin in the soluble fraction was due to dilution of the radioactive albumin from the microsomes with the inactive albumin in the soluble fraction. This seems unlikely, however, for in the small-scale experiments the microsomal rat albumin had such a high radioactivity that even if it had been diluted with twice the quantity of inactive albumin it would have remained significantly radioactive. Thus the results of the present experiments suggest that the albumin synthesized in the microsomes during incubation is not released in soluble form.

Simkin (1958) has studied the release of soluble proteins from the microsomal fraction of guinea-pig liver incubated under conditions similar to those of the present experiments. He found that the soluble proteins were released from the microsomes only under conditions in which amino acid incorporation took place. Since it was not possible to uncouple the release from the incorporation it was not clear whether the passage of protein from the microsomes to the soluble fraction was the result of an active process, i.e. secretion, or was merely a displacement of protein from the reticulum due to the formation of new protein. In view of these results involving a mixture of soluble proteins it was a surprise to find that in the present experiments serum albumin was not transferred from the microsomes to the soluble fraction. Since albumin was being synthesized in the microsomes the most likely explanation is that the transfer of albumin from the microsomes to the soluble fraction is an active process and not a displacement. Schweet *et al.* (1958), in their studies on the incorporation of [^{14}C]amino acids into haemoglobin by the microsome fraction from rabbit reticulocytes, isolated radioactive haemoglobin from the soluble fraction of the incubation mixture. Thus in this case the haemoglobin presumably was released from the microsomes during incubation. The difference between the two systems may be that whereas albumin is synthesized by the liver for 'export', haemoglobin is synthesized by the reticulocyte for its own requirements. Variations in the morphology of the two types of microsomes may also account for the difference.

Since this paper was prepared Jungblut, Heimburger & Turba (1959) have reported that four proteins with antigenic properties against an antiserum prepared against purified rat albumin may be extracted from rat-liver microsomes by deoxycholate. Only one of these proteins has the electrophoretic properties of serum albumin. The electrophoretic method described above for the isolation of albumin from the radioactive microsomes clearly separated serum albumin and α_1-globulin. Hence the conclusions reached in the present paper are not affected by the findings of Jungblut *et al.* (1959).

SUMMARY

1. The microsomal fraction from regenerating rat liver was incubated with radioactive amino acids, adenosine triphosphate, phosphoenolpyruvate and cell sap. The microsomes were separated from the soluble proteins and were disintegrated with ultrasonics. The rat albumin released from the microsomes and in the soluble fraction of the incubation mixture was precipitated by the addition of an antiserum and its radioactivity was determined. The results indicated that there was an incorporation of amino acids into the microsomal albumin but not into the albumin in the medium.

2. The rat albumin isolated from the microsomes and the medium after incubation as described above was purified by the trichloroacetic acid–ethanol method and then subjected to electrophoresis on strips of cellulose acetate paper. The microsomal rat albumin retained its radioactivity throughout the course of these purification procedures.

3. Similar results were obtained with microsomes from normal liver.

4. Identical isolation procedures were applied to albumin isolated from liver microsomes 20 min. after injecting rats with radioactive amino acids. The radioactivity of the rat albumin was determined throughout the course of isolation.

5. A comparison of the radioactivity of the insoluble microsomal protein and the microsomal albumin in the experiments *in vitro* and *in vivo* showed that the isolated microsomes suffered a relatively greater loss in their ability to synthesize albumin than insoluble protein.

6. It is concluded that the results are consistent with the hypothesis that isolated rat-liver microsomes are able to synthesize albumin but that this albumin is not released in a soluble form.

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Metabolism of Polycyclic Compounds

15. THE CONVERSION OF NAPHTHALENE INTO A DERIVATIVE OF GLUTATHIONE BY RAT-LIVER SLICES*

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Naphthalene is converted by animals into a compound which is believed to be *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (scheme 1, III), which gives rise to 1-naphthylmercapturic acid (VI) on acidification (Boyland & Sims, 1958). Knight & Young (1958) found that benzene, anthracene and the monohalogenobenzenes were probably converted into analogous derivatives. The mode of formation of these acids in the body has not yet been established, but they may arise by the reaction of intermediates (such as epoxides) with an SH-containing compound. In the formation of mercapturic acids, cysteine, glutathione and the cysteine SH of tissue protein have all been cited

* Part 14: Sims (1959).

from time to time by various workers as the source of the cysteine residues. This earlier work has recently been reviewed by Barnes, James & Wood (1959), who have found that when a number of compounds which are known to give rise to mercapturic acids are fed to rats, the levels of glutathione in the livers decrease in amounts roughly commensurate with the quantities of mercapturic acids formed. On the other hand, the glutathione levels in the blood, kidney and intestine are unaffected.

The present work, in which naphthalene has been incubated *in vitro* with rat-liver slices, has shown that a substance which is believed to be *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I) is formed.

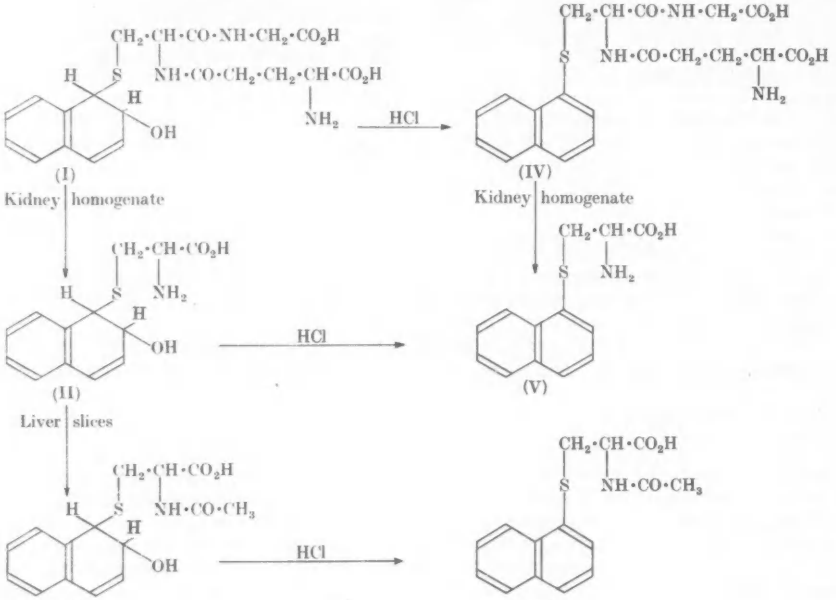
METHODS AND MATERIALS

Melting points are uncorrected.

S-(1-*Naphthyl*)*glutathione* (IV). This compound was prepared by a modification of the method of Parke & Williams (1951). *Glutathione* (4 g.) (which was known to contain a little of the oxidized form) in water (100 ml.) and acetic acid (5 ml.) was warmed to 50–60° with zinc dust (3 g.) for 15 min. and then kept at room temperature with occasional shaking for 3 hr. Cuprous oxide was added until no more would dissolve and the solution was cooled to 0° and stirred while 1-naphthylamine (2 g.) diazotized in N-H_2SO_4 (50 ml.) with $NaNO_2$ (1 g.) was slowly added. The mixture was stirred at 0° for 1 hr. and at room temperature for 1 hr. and was then kept overnight at 0°. The brown precipitate was filtered off and the filtrate was washed with ether (3 × 250 ml.). Activated charcoal (10 g.) was added to the aqueous layer and the charcoal was filtered off and washed with water (1 l.). The adsorbed material was eluted with methanol (500 ml.) containing 5% (v/v) of aq. NH_3 soln. (sp.gr. 0·88) and the solution was evaporated to small volume under reduced pressure. The residue was acidified with acetic acid and the precipitated solid was recrystallized from water to yield *S*-(1-*naphthyl*)*glutathione* (IV) (1·2 g.) as a cream-coloured powder, m.p. 225–226° (decomp.) (Found: C, 55·1; H, 5·4; N, 9·85; S, 7·2; $C_{20}H_{23}O_6N_3S$ requires C, 55·4; H, 5·35; N, 9·7; S, 7·4%). A little of the compound was hydrolysed by heating to 100° with 6 N-HCl for 2 hr. and the products were examined on paper chromatograms with solvent 3 (Table 1). Glycine, glutamic acid and *S*-(1-naphthyl)cysteine were detected as ninhydrin-positive spots at R_F 0·23, 0·28 and 0·71 respectively.

S-(1-*Naphthyl*)-L-*cysteine* (V). This was prepared by the method of West & Mathura (1954), except that the compound was isolated from the reaction mixture by means of activated charcoal as outlined above. It crystallized from water in needles, m.p. 184° (decomp.), $[\alpha]_D^{25}$ +6° in 0·1 N-NaOH (*c*, 1).

1-*Naphthylmercapturic acid* (VI). This was prepared by the acetylation of *S*-(1-naphthyl)-L-cysteine in 2 N-NaOH with acetic anhydride and formed needles from water, m.p. 173°, $[\alpha]_D^{25}$ −24° in ethanol (*c*, 1).



Scheme 1

N-Acetyl-S-(1:2-*dihydro*-2-*hydroxy*-1-*naphthyl*)-L-*cysteine* (III). This was isolated as the ammonium salt from the urine of rabbits dosed with naphthalene as described previously (Boyland & Sims, 1958).

Paper chromatography

Descending chromatograms on Whatman no. 1 chromatography paper were developed for 18 hr. with the solvents listed in Table 1. The dried chromatograms were examined in u.v. light after exposure to NH_3 and were then sprayed with one of the following reagents: 1, diazotized *p*-nitroaniline (0·02% in 0·1 N-HCl) followed by aq. 10% (w/v) Na_2CO_3; 2, 0·1 M-$K_2Cr_2O_7$–acetic acid (1:1) followed by 0·1 M-$AgNO_3$ (Knight & Young, 1958); 3, a solution of ninhydrin (0·4%) in butanol saturated with water. Other chromatograms were sprayed with 2 N-HCl and heated to 70° for 10 min. before being sprayed with reagent 1 above.

Tissue experiments

Slices of liver (approx. 5 g. wet wt.) from male adult rats were incubated in Ringer phosphate solution (Umbreit, Burris & Stauffer, 1949) saturated with naphthalene (final concn. approx. 0·3 mM) pH 7·4 (60 ml.) for 2 hr. at 37° with shaking. At the end of the incubation period the reaction mixture was decanted from the slices, which were washed once with water (30 ml.). The washings and the reaction mixture were combined and adjusted to pH 4·5 with acetic acid. The reaction mixture was centrifuged for 20 min. at 600 *g*, the supernatant was treated with a little activated charcoal and the charcoal filtered off and washed with water. The material obtained by the subsequent elution of the charcoal with methanol containing 0·2% (v/v) of aq. NH_3 soln. (sp.gr. 0·88) and evaporation under reduced pressure was examined on paper chromatograms. Kidneys from male adult rats were homogenized in 9 vol. of 0·154 M-NaCl with a Potter & Elvehjem (1936)-type homogenizer with a Teflon pestle.

RESULTS

Identification of the new metabolite

Examination of the products from the incubation of naphthalene with liver slices on paper chromatograms showed the presence of a compound which formed a dark absorbent spot when the chromatograms were inspected in u.v. light, gave a blue on spraying with diazotized *p*-nitroaniline and aq. Na_2CO_3, a mauve with ninhydrin and a positive test with the $K_2Cr_2O_7$–$AgNO_3$ reagent. On two-dimensional chromatograms, run in either solvent system 1 or 2, which had been sprayed with aq. N-HCl and allowed to dry between the first and second developments, the compound could no longer be detected, but a second compound of different R_F was seen, which had a bright-orange fluorescence under u.v. light and reacted with ninhydrin and the $K_2Cr_2O_7$–$AgNO_3$ reagent but not with diazotized *p*-nitroaniline. At the same time a spot identical with that of 1-naphthol was formed. The orange fluorescent spot was indistinguishable from synthetic *S*-(1-naphthyl)glutathione (IV) on paper chromatograms. The properties (apart from the ninhydrin reaction) of the original compound were similar to those of *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (III), except that this compound forms 1-naphthylmercapturic acid on acidification (Boyland & Sims, 1958) and has a higher R_F in all the solvents used (see Table 1). These properties suggested that the new metabolite was *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I).

Approximately 25 g. of liver slices was incubated with Ringer phosphate saturated with naphthalene as described above. The product was prepared for paper chromatography and the solution applied as a 15 cm. long strip to the paper, which was then developed with solvent 1 (Table 1) for 18 hr. The chromatogram was dried and a test strip cut off and sprayed with diazotized *p*-nitroaniline and aq. Na_2CO_3. The zone on the main chromatogram containing the metabolite was cut out and eluted with

Table 1. *Paper chromatography of compounds related to* S-(1:2-*dihydro*-2-*hydroxy*-1-*naphthyl*)*glutathione*

Solvent systems: 1, butanol–propan-1-ol–aq. 2 N-NH_3 soln. (2:1:1, by vol.); 2, butanol–acetic acid–water (2:1:1, by vol.); 3, the organic phase of butanol–acetic acid–water (5:1:4, by vol.). Time of run, 18 hr. All compounds gave positive reactions with the $K_2Cr_2O_7$–$AgNO_3$ reagent.

Compounds	R_F Solvent 1	R_F Solvent 2	R_F Solvent 3	Fluorescence	Colour: With diazotized *p*-nitroaniline and Na_2CO_3	Colour: With ninhydrin
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-glutathione	0·04	0·47	0·36	Dark-absorption	Blue	Mauve
S-(1:2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0·31	0·63	0·53	Dark-absorption	Blue	Mauve
N-Acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0·37	0·84	0·82	Dark-absorption	Blue	—
S-(1-Naphthyl)glutathione	0·07	0·69	0·53	Orange	—	Mauve
S-(1-Naphthyl)-L-cysteine	0·46	0·75	0·69	Orange	—	Mauve
1-Naphthylmercapturic acid	0·51	0·86	0·87	Orange	—	—

methanol containing 0·2% of aq. NH_3 soln. (sp.gr. 0·88). The solution was evaporated to a small volume and the residue applied as a 5 cm. long strip to a chromatogram which was then developed with solvent 2 (Table 1). The metabolite was located on the dried chromatogram by means of its dark absorption in u.v. light, and this portion of the chromatogram was cut out and eluted, and the eluate evaporated as before. The material thus obtained was chromatographed on a two-dimensional chromatogram with solvent 3 (Table 1), the chromatogram being sprayed with N-HCl and allowed to dry before the second development with solvent 3. The spot with an orange fluorescence under u.v. light was cut out and the material eluted with methanol–aq. NH_3 soln. as before. In this way a solution of the acid-decomposition product of the metabolite free from contamination by other substances (particularly amino acid-containing substances) was obtained. The methanol was distilled off and the residue heated to 100° with HCl (0·5 ml.) for 3 hr. The solution was evaporated to dryness, the residue was dissolved in water (0·5 ml.) and the solution again evaporated to dryness. The residue was treated with 1-fluoro-2:4-dinitrobenzene as described by Sanger (1945) and the material thus obtained examined on paper chromatograms with the conditions and solvents of Phillips (1958). By this means, three dinitrophenyl derivatives were detected, identical with those of glycine, glutamic acid and *S*-(1-naphthyl)-L-cysteine.

Enzymic decomposition

A solution (100 ml.) of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I), obtained from incubation of 5 g. of liver slices as indicated above, except that the elutions were carried out with Ringer phosphate, was incubated with kidney homogenate (10 ml.) at 37° for 2 hr. and treated with charcoal. Examination of the products on paper chromatograms showed that the glutathione derivative (I) was no longer present in the reaction mixture. Instead a compound was present which showed as a dark absorbent spot in u.v. light and gave the colour reactions of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione, but which differed from this compound in its R_F values in all the solvents used. On two-dimensional chromatograms with solvent 2, which were sprayed with N-HCl and dried between the first and second developments, the original compound could no longer be detected, but a compound which was indistinguishable from *S*-(1-naphthyl)-L-cysteine (V) was detected together with 1-naphthol. The above evidence suggests that the new compound is *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (II).

When a solution of this metabolite, obtained by elution of the appropriate portion from a paper chromatogram with Ringer phosphate (100 ml.), was incubated with liver slices (approx. 5 g.) its acetylated derivative (III) could be identified in the reaction mixture. Furthermore when a solution of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione in boiled liver extract, obtained by boiling the supernatant from the incubation of liver slices with naphthalene, was incubated with kidney homogenate both *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine and *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine were identified by paper chromatography.

S-(1-Naphthyl)glutathione (2 mg.) in Ringer phosphate (100 ml.) and rat-kidney homogenate (10 ml.) were incubated together at 37° for 2 hr. The product, separated from the reaction mixture with charcoal as before, was shown on paper chromatograms to be indistinguishable from *S*-(1-naphthyl)-L-cysteine.

None of the above products was detected in the reaction mixture when naphthalene was incubated with boiled liver slices, nor were they formed by liver slices in the absence of naphthalene. Boiled kidney homogenate was unable to effect the hydrolysis of the glutathione derivative (I). In the experiments in which naphthalene was incubated with liver slices, other naphthalene metabolites were detected on paper chromatograms run in solvent 1 (Table 1) by means of the colour reactions with diazotized *p*-nitroaniline and aq. Na_2CO_3 described by Sims (1959). These included *trans*-1:2-dihydro-1:2-dihydroxynaphthalene, 1-naphthyl sulphate, 1-hydroxy-2-naphthyl sulphate and *trans*-1:2-dihydro-2-hydroxy-1-naphthylglucosiduronic acid.

DISCUSSION

Although the problem of mercapturic acid excretion by animals has been studied over many years by different workers, there is little agreement about the mechanism by which these acids are formed, although some reaction of the foreign compound with the SH groups of cysteine, glutathione or tissue protein seems probable. There are two aspects to this problem: the mechanism of the reaction of the foreign compound with SH groups and the nature of the residue of which the SH group is part. Barnes *et al.* (1959) have divided compounds which are known to give rise to mercapturic acids into types according to the group which is displaced by *N*-acetylcysteine in the formation of these acids. Apart from the aromatic hydrocarbons and the halogenobenzenes, the compounds contain groups which could react *in vitro* under suitable conditions with *N*-acetylcysteine to form mercapturic acids. The aromatic hydrocarbons and the halogenobenzenes possess no such reactive

groups, but the formation of reactive intermediates such as epoxides (Boyland, 1950; Boyland & Sims, 1958), which then react with SH-containing compounds, could explain the formation of compounds such as *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (VI) in the body. Benzene, anthracene and the halogenobenzenes give similar acid-labile metabolites (Knight & Young, 1958).

There is little doubt that the product formed when naphthalene is metabolized by liver slices is related to the product in the urine of animals dosed with naphthalene and differs from it in having a glutathione residue in place of an acetylcysteine residue. If, as seems likely, the product from the urine has the structure (III) (Boyland & Sims, 1958), then the product from the liver slices is *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I) and this structure is confirmed by the formation of *S*-(1-naphthyl)glutathione (IV) with cold acid and of glycine, glutamic acid and *S*-(1-naphthyl)-L-cysteine (VI) with hot acid. Other amino acids were not detected. The product formed by the enzymic hydrolysis of the glutathione derivative (I) by kidney homogenates is also clearly related to the product from the urine and, since it gives *S*-(1-naphthyl)-L-cysteine (V) on acid decomposition, it is presumed to be *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (II). This compound is acetylated by liver slices to yield *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (III), identical with that from the urines of naphthalene-treated animals. Neither the glutathione derivative (I) nor the cysteine derivative (II) has been detected in these urines: evidently only the acetyl derivative is excreted. It is generally accepted that the final stage in mercapturic acid synthesis involves an acetylation of the amino group of the substituted cysteine. West & Mathura (1954), for example, found that *S*-substituted cysteines are converted by animals into the mercapturic acids, and *S*-(*p*-bromophenyl)-L-cysteine is acetylated by liver and kidney slices (Gutmann & Wood, 1951). In experiments in which [^{131}I]iodobenzene was fed to rats, Mills & Wood (1956) found that liver, kidney and intestine contained *S*-(*p*-iodophenyl)-L-cysteine, whereas only the mercapturic acid was excreted in the urine. When mercapturic acids are fed to animals large proportions are excreted unchanged (cf. Marsden & Young, 1958). Stekol (1941) showed that *S*-(*p*-bromobenzyl)glutathione is excreted as *p*-bromobenzylmercapturic acid when fed to rats, and Roberts & Warwick (1958) showed that *S*-ethylglutathione is converted into ethylmercapturic acid by these animals.

The conversion of the glutathione derivative (I) into the cysteine derivative (II) by kidney homogenate could have been brought about either by the loss of glycine and glutamic acid or by the replacement of the glutathione moiety by cysteine. The former reaction, which could involve either a hydrolysis or a transfer of the amino acids to acceptors, seems the more likely. With glutathione itself, cleavage can take place either by hydrolysis or by transfer and these reactions are carried out by kidney and, according to some workers, by liver. Binkley & Nakamura (1948) found, for example, that only rat kidney converted glutathione into cysteinylglycine whereas other tissues could break down the latter compound. Revel & Ball (1959) showed that of 12 rat tissues, including liver, only kidney and pancreas could transfer the glutamyl group of glutathione. On the other hand, Neubeck & Smythe (1944) found that guinea-pig liver could hydrolyse glutathione with the production of cysteine and Cliffe & Waley (1959) found that the nuclear fraction of rabbit liver could catalyse both the hydrolysis and transpeptidation of glutathione. Bray, Franklin & James (1959) found that guinea-pig-liver slices and homogenates of guinea-pig, rat and rabbit liver would convert *S*-(*p*-chlorobenzyl)-glutathione into *S*-(*p*-chlorobenzyl)-L-cysteine, whereas in the present work the conversion of naphthalene into *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (II) via the glutathione derivative (I) could not be effected by liver slices alone, even on prolonged incubation, although it might be that small amounts of the decomposition products would not be detected by the methods used. It seems probable therefore that, in animals treated with naphthalene, the glutathione derivative (I) is formed in the livers and is broken down to the cysteine derivative (II) in the kidneys. The conversion of this into the mercapturic acid (III) could take place in either of these organs. Mills & Wood (1954) showed that the products from the incubation of [^{131}I]iodobenzene with rat liver (and kidney) slices were mainly iodophenol derivatives, although a little mercapturic acid was formed, whereas in intact animals this acid is predominant. In later experiments (Mills & Wood, 1956) small amounts of *S*-(*p*-iodophenyl)-L-cysteine were also found. It is possible that in these experiments, in which the labelled metabolites were isolated by extraction from acidified solutions and purified and identified by the use of carrier acids, a glutathione derivative similar to the one found in the present work was also formed but not detected.

The conversion of naphthalene into the glutathione derivative (I) could have occurred by the direct action of an intermediate on glutathione, and the work of Barnes *et al.* (1959) suggests that this might be so. On the other hand, Mills & Wood (1956) isolated a protein apparently containing radioactive material when the rat was fed [^{131}I]-iodobenzene, so that protein (or large peptide) SH groups may be involved. If this is so, then either

the protein must contain glutathione residues which are eliminated after the reaction with the intermediate or a cysteine derivative is eliminated which then reacts with glycine and glutamic acid to form the glutathione derivative. If it is cysteine which reacts with the intermediate, then a similar synthesis of the glutathione derivative must be envisaged. Glutathione can be formed in the body by a two-step addition of glutamic acid and glycine to cysteine (Meister, 1957), but analogous reactions seem unlikely to occur in the biosynthesis of the naphthalene derivative; substituted dipeptides such as γ-glutamyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine have not been encountered, whilst the incubation of *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine (II) with liver slices did not yield *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (I). At the moment therefore it seems likely that glutathione is the peptide involved in mercapturic acid synthesis, but the possibility that reaction with a larger peptide or a protein may occur is under investigation.

Even if the main reaction is with glutathione, it is possible that some reaction with protein SH groups occurs, but that the substituted cysteine residues are not subsequently eliminated from the protein. It may be that the polynuclear hydrocarbons, which are not excreted as mercapturic acids (Crabtree, 1946; Gutmann & Wood, 1950), behave in this way. Alternatively, it may be that these compounds form glutathione derivatives, similar to the one described in this paper, which cannot then be split in the body and are therefore not eliminated.

SUMMARY

1. *S*-(1-Naphthyl)glutathione, *S*-(1-naphthyl)-L-cysteine and 1-naphthylmercapturic acid have been synthesized.

2. Slices of livers of male rats convert naphthalene into a compound which is almost certainly *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione and which yields *S*-(1-naphthyl)glutathione when treated with acid at room temperature.

3. Rat-kidney homogenates convert *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione into a compound believed to be *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine and which forms *S*-(1-naphthyl)-L-cysteine on treatment with acid at room temperature. Kidney homogenates also hydrolyse *S*-(1-naphthyl)glutathione to *S*-(1-naphthyl)-L-cysteine.

4. *S*-(1:2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteine is acetylated by liver slices or kidney homogenate and boiled liver extract to *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine.

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The Distribution of Phospholipase B in Mammalian Tissues

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The breakdown of phospholipids in animal tissues has for long been regarded as involving the removal of the fatty acid residues by the successive action of phospholipases A and B, followed, in lecithin breakdown, for example, by the degradation of the glycerylphosphorylcholine so formed by means of a specific diesterase. The initial removal of a single fatty acid from the phospholipid molecule results in the formation of a lysophosphatide. Because of the marked cytolytic properties of these compounds, together with the fact that lysolecithin has recently been shown to be present in lipid extracts of animal tissues (Marinetti, Witter & Stotz, 1957; Hadju, Weiss & Titus, 1957; Phillips, 1957, 1958), a comparison of the ability of different tissues to degrade the lysophosphatides by means of phospholipase B is of physiological interest.

Although this enzyme was first recognized in rice bran by Contardi & Ercoli in 1933, and was found also to occur in certain moulds (Fairbairn, 1948) and bacteria (Hayaishi & Kornberg, 1954), little detailed work appears to have been done on its distribution in animal tissues. Noguchi (1944) studied a number of rabbit tissues, and found considerable phospholipase B activity in pancreas and lung. Shapiro (1953) obtained active preparations from glycerol extracts of ox pancreas, and Dawson (1956) has carried out a careful study of the properties of this enzyme in glycerol extracts of acetone-dried rat and sheep liver. Dawson also states that he obtained liberation of glycerylphosphorylcholine when glycerol extracts of rat kidney, spleen, brain and blood were incubated with lysolecithin, but no quantitative assessment of enzymic levels was given for these tissues.

While this paper was in preparation a comparison of some of the properties of the phospholipase B in rat intestinal mucosa with those of the lecithinase also present in this tissue has been published by Epstein & Shapiro (1959).

EXPERIMENTAL

Tissue preparations. Homogenates (1 g. of tissue in 4 ml. of water) of the various tissues were prepared by using a modified Potter–Elvehjem homogenizer (Webster, 1954). In some cases the tissue was finely minced with sharp scissors before homogenization. Rat skeletal muscle was homogenized for 5 min. in a macerator (M.S.E., Ltd.), after first mincing finely with scissors.

Rat tissues were removed from freshly decapitated animals, and rabbit tissues from animals freshly killed with pentobarbital sodium (60 mg./kg. body wt.). Human brains were obtained from cadavers as soon as possible after death; the areas of brain to be investigated were dissected, washed with aq. 0·9% NaCl and homogenized as above.

Estimation of enzymic activity. Activity of the homogenates was estimated by determination of both free fatty acid release and of the glycerylphosphorylcholine (GPC) formed after incubation with lysolecithin.

Samples (2·0 ml.) of homogenates prepared as above were incubated at 38° with 2·5 ml. of 0·2 M-KH_2PO_4–Na_2HPO_4 buffer (pH 6·5) and 6·2 mM-lysolecithin in a final volume of 5·0 ml.

Estimation of free fatty acids. For the estimation of free fatty acid release, a 2·0 ml. sample was removed from the reaction mixture immediately after the addition of the lysolecithin, and a further 2·0 ml. sample removed after incubation for 60 min. at 38°. Estimations were also carried out on samples from control tubes from which either tissue or lysolecithin had been omitted. The method of van der Vies (1955) was used; this involves extraction with light petroleum (40–60°) and, after removal of the light petroleum, titration with 0·01 N-NaOH by using a Conway horizontal burette.

Estimation of glycerylphosphorylcholine. The estimation of GPC involves precipitation of protein with trichloroacetic acid followed by determination of free choline before and after hydrolysis by heating in N-HCl at 100° for 20 min. (Schmidt, Hecht, Fallot, Greenbaum & Thannhauser, 1952). Since Dawson (1956) had pointed out that lysolecithin is also hydrolysed under these conditions, and since lysolecithin is only partially precipitated with the protein, the fraction remaining in solution would interfere with the estimation of GPC. To overcome this difficulty, lysolecithin was added to the control tubes (tissue only) at the end of the 60 min. incubation period and immediately before the addition of the trichloroacetic acid. As a further precaution against interference from unprecipitated lysolecithin the samples, after the addition of trichloroacetic acid, were frozen solid and centrifuged immediately after thawing (this procedure was found to increase the proportion of lysolecithin precipitated); after removal of the precipitated protein, the still cloudy solutions were incubated at 38° with bovine γ-globulin and reprecipitated with trichloroacetic acid; by this procedure clear supernatants were obtained.

GPC was determined by estimating free choline, before and after hydrolysis (see above), with the periodide method of Appleton, La Du, Levy, Steele & Brodie (1953), as modified by Webster (1956).

A 2·0 ml. sample of the reaction mixture was pipetted into 1·0 ml. of 15% (w/v) trichloroacetic acid solution. After mixing, the contents of the tubes were frozen solid

and, after being allowed to melt, were immediately centrifuged for 10 min. at 1700 *g*. A sample of the supernatant (2·0 ml.), which varied from clear to very cloudy, was extracted twice with ether; after removal of the last traces of ether by bubbling through nitrogen, the solution was incubated for 5 min. at 38° with 0·4 ml. of a 5% (w/v) suspension of bovine γ-globulin (Armour). Trichloroacetic acid solution (1·2 ml. of 15%, w/v) was added and the precipitated proteins were removed by centrifuging. A portion (0·5 ml.) of the clear supernatant was used for estimation of free choline, and 1·5 ml. was heated in a firmly stoppered tube for 20 min. at 100° with 0·5 ml. of 4 N-HCl. After cooling, 0·5 ml. of the hydrolysate was used for estimation of total (free + acid-labile) choline.

Standards and blanks were set up as follows: after incubation of homogenate with buffer, GPC and lysolecithin were added to the standard tubes, and lysolecithin only to the blank tubes, trichloroacetic acid being added to each immediately after these additions.

Lysolecithin. This was prepared from ovolecithin by the method of Long & Penny (1957). The crude lysolecithin was crystallized from hot ethanol (Saunders & Thomas, 1958), which removed phospholipase A precipitated with the lysolecithin during the preparation (Marples & Thompson, 1958).

Glycerylphosphorylcholine. The crystalline $CdCl_2$ complex of GPC was prepared by Dr G. R. Webster in this Laboratory according to the method of Tattrie & McArthur (1955). Aqueous solutions of free GPC were freshly prepared from this by removal of the cadmium on an ion-exchange resin (Zeo-Karb 225, Na form).

Buffers. For the pH–activity curve, sodium acetate–acetic acid buffers at pH 5·0 and 5·5, and KH_2PO_4–Na_2HPO_4 buffers at pH 6·0, 6·5, 7·0 and 7·4, were used, all at a final concentration of 0·1 M.

RESULTS

Although Dawson (1956) has described the properties of the phospholipase B in glycerol extracts of acetone-dried preparations of rat liver, it was thought advisable to characterize briefly some of the properties of this enzyme in fresh homogenates of whole liver. When rat-liver homogenate and lysolecithin were incubated together at pH 6·5, there was a release of free fatty acid amounting to 29·4 μmoles/g./hr., together with a production of GPC of 31·5 μmoles/g./hr., but no formation of free choline. The reaction proceeded rapidly for at least 1 hr., though the rate fell off slowly throughout the course of the reaction.

A series of buffers with pH values between 5·0 and 7·4 were used to determine the optimum pH for the enzyme. Rat-liver homogenate was incubated for 60 min. at 38° with 6·2 mM-lysolecithin by using these buffers, and the release of free fatty acid was determined. The results are given in Fig. 1 and show that the optimum pH is at 6·5, although there is considerable activity at the physiological pH.

The effect of enzyme concentration and substrate concentration on the rate of reaction was studied. The rate of reaction increased linearly with increasing weights of liver between 100 and 400 mg. of tissue/5 ml. of reaction mixture; the rate also increased with increasing substrate concentration up to a maximum at 6·2 mM (Fig. 2). No evidence was found of any falling-off of activity at the higher substrate concentrations studied. From the substrate concentration–activity curve a Michaelis constant of 2·3–2·6 mM was calculated.

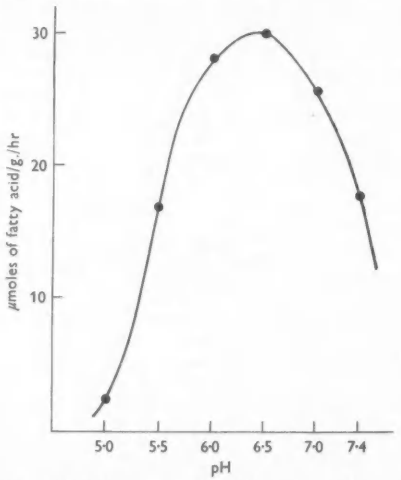

Fig. 1. pH–Activity curve of rat-liver phospholipase B.

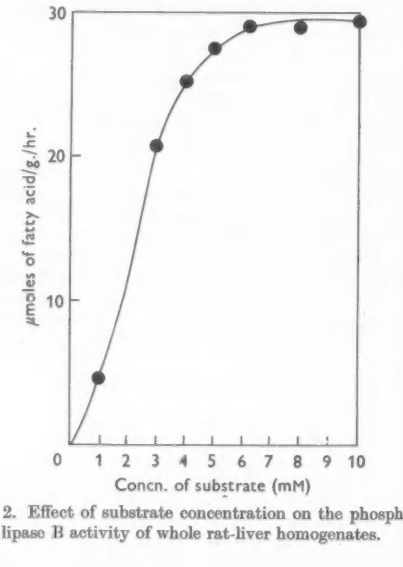

Fig. 2. Effect of substrate concentration on the phospholipase B activity of whole rat-liver homogenates.

Table 1. *Effect of some inhibitors on phospholipase B activity of homogenates of whole rat liver*

Inhibitor	Concn. (mM)	Activity (μmoles of fatty acid/g./hr.) Normal	Inhibited	Inhibition (%)
EDTA	1	16·6	16·9	0
NaF	20	16·6	9·6	42
KCN	10	16·6	13·3	20
Cysteine	10	16·6	17·2	0
Hg^{2+}	1	22·4	0	100
Zn^{2+}	1	30·7	19·8	33
Cu^{2+}	1	30·3	2·8	90
Mg^{2+}	1	20·9	18·1	13
Iodoacetate	1	15·6	15·4	0

Table 2. *Distribution of phospholipase B in homogenates of whole rat tissues*

Here and in Table 3 activities expressed as μmoles of free fatty acid or of GPC + free choline formed/g./hr. and the number of experiments is given in parentheses.

Tissue	Activity (μmoles/g./hr.) Fatty acid	Total choline	Free choline	GPC
Lung	57·2 (2)	51·6 (2)	7·1 (2)	44·5
Ileum	71·2 (3)	72·3 (2)	2·6 (2)	69·7
Spleen	52·3 (2)	46·7 (4)	3·2 (4)	43·5
Liver	29·4 (5)	31·5 (4)	0 (3)	31·5
Pancreas	27·2 (3)	—	—	—
Kidney	9·4 (2)	9·6 (2)	4·4 (2)	5·2
Testis	6·7 (2)	5·3 (2)	0 (2)	5·3
Skeletal muscle	4·4 (2)	5·6 (2)	0·1 (2)	5·5
Heart	—	2·1 (2)	0 (2)	2·1
Brain	—	1·7 (3)	0 (3)	1·7
Spinal cord	—	0·8 (2)	0 (2)	0·8
Adipose tissue	0 (2)	—	—	—

Table 3. *Phospholipase B activity of some areas of human brain*

	Activity (μmoles/g./hr.) Fatty acid	Total choline	Free choline	GPC
Grey matter	3·9 (2)	6·4 (2)	0 (2)	6·4
White matter	0·7 (2)	0·3 (2)	0 (2)	0·3
Cerebellum	4·8 (3)	3·9 (2)	0 (2)	3·9
Caudate nucleus	5·0 (2)	5·4 (2)	0·8 (2)	4·6
Corpus callosum	1·9 (2)	0·8 (2)	0 (2)	0·8

The phospholipase B activity of whole-liver homogenates is sensitive to heat; incubation of a water homogenate of rat liver for 15 min. at 60° completely abolished the enzymic activity.

Effect of inhibitors. Solutions of the inhibitors tested were added to the liver homogenate and buffer, and the mixture was incubated for 10 min. at 38° before the addition of lysolecithin. Unlike the phospholipase A of cottonmouth-moccasin venom (Long & Penny, 1957), the liver phospholipase B was unaffected by mM-ethylenediaminetetra-acetic acid (EDTA); Dawson (1956) had found that the phospholipase B of an extract of acetone-dried rat liver was slightly activated by this concentration of EDTA. The effect of various metal ions on the enzymic activity was tested and the results are shown in Table 1. Ca^{2+} and Mn^{2+} ions (mM) had no significant effect. The enzyme was unaffected by cysteine (10 mM) and by iodoacetate (mM); 20 mM-NaF and 10 mM-KCN produced inhibitions of 42 and 20% respectively.

Distribution of enzyme. The distribution of phospholipase B has been studied in a variety of rat tissues and in some areas of human brain. The results are presented in Tables 2 and 3, where the amounts of free fatty acid, GPC and choline formed/g. of tissue/hr. are given. Intestine (ileum), lung and spleen were the most active of the rat tissues tested, while nervous tissue and heart had little activity. No values have been given for free fatty acid release from brain (rat), spinal cord and heart, as the activity of these tissues was too low for accurate estimation by the method used; it was not possible to estimate the GPC and free choline formed in rat pancreas, because this tissue contains a substance which forms a copious precipitate with the iodine reagent, and though this was only slightly soluble in chloroform it had a high extinction at 365 mμ. The results with human brain (Table 3) suggest that the activity in this tissue is associated with the grey rather than with the white matter. Human blood showed only very low levels

of activity, serum and packed red cells releasing 0·51 and 0·83 μmole of fatty acid/ml./hr. respectively.

Noguchi (1944) has described the phospholipase B activity of extracts of rabbit lung and pancreas, but found no activity in extracts of rabbit kidney, spleen, intestine or liver. With fresh preparations, however, measurable activity was found in all these rabbit tissues; the activity was of the same order as that of rat tissues, with the exception of intestine, which had a relatively low activity, and brain, which was more active than rat brain. Rabbit grey matter was more active than rabbit white matter.

It has been mentioned above that there was no formation of free choline during the incubation of lysolecithin with rat-liver homogenate at pH 6·5. This finding agrees with that of Dawson (1956), who noted that rat-liver GPC-diesterase was inactive at pH 6·5. As can be seen in Table 2, however, when rat kidney, intestine, spleen and lung were incubated with lysolecithin at pH 6·5 there was a measurable production of free choline; with rat kidney almost half the total acid-soluble choline produced was present as free choline. This could be explained if rat kidney contained a GPC-diesterase with activity at pH 6·5. Rat-kidney homogenate was therefore incubated at 38° and pH 6·5 with 0·3 mM-GPC (the concentration of GPC formed from lysolecithin by rat kidney in 60 min.). Free choline was formed at the rate of 7·6 μmoles/g./hr., and the diesterase activity is therefore probably sufficient to account for the production of choline from the GPC formed by the phospholipase B. With 0·02 M-GPC, the GPC-diesterase activity of rat kidney (32·3 μmoles of choline produced/g./hr.) was about four times as much at pH 6·5 as that of rat brain (Webster, Marples & Thompson, 1957).

DISCUSSION

The results described above indicate that phospholipase B activity is readily demonstrable in simple water homogenates of a number of mammalian tissues. Titration of the fatty acid released, or estimation of the glycerylphosphorylcholine formed, after incubation of the homogenate with lysolecithin provides a satisfactory method for the estimation of enzymic activity, and the agreement between the estimates obtained by these two analytical methods is reasonable (Table 2).

A comparison of our findings on whole ileum (i.e. mucosa and muscle layers) with those of Epstein & Shapiro (1959) on intestinal mucosa suggests that the greater part of the intestinal activity is associated with the mucosa. Hajdu *et al.* (1957) have recently identified the substance with digitalis-like activity, which occurs in mammalian blood, as β-palmitoyl-lysolecithin; rat-heart muscle contains even less phospholipase B than skeletal muscle.

In general, the properties of the phospholipase B, as displayed in whole-tissue homogenates (liver), appear to agree well with those described by Dawson (1956) for the partially purified enzyme in the glycerol extracts of acetone-dried rat liver which he used. The only major difference which we have found is in the effect of substrate concentration. Dawson found maximal activity with substrate levels between 2 and 3·5 mM, with a reduction of activity at higher substrate concentrations; using whole-tissue homogenates we have found maximal activity with about 6 mM-lysolecithin, and no fall-off in the rate with increases of substrate concentration up to 10 mM. Epstein & Shapiro (1959), using aqueous preparations of rat intestinal mucosa, have also found no fall-off in the rate with concentrations up to 10 mM-lysolecithin, although the glycerol extracts of acetone-dried ox pancreas used earlier by Shapiro (1953) showed an effect comparable with that found by Dawson (1956).

SUMMARY

1. A study has been made of the distribution of phospholipase B in certain mammalian tissues, by using whole homogenates of the different tissues.

2. Enzymic activity has been estimated by determining the rate of release of both free fatty acids and glycerylphosphorylcholine.

3. The properties of the enzyme in whole rat-liver homogenates agree closely with those described by Dawson (1956) in glycerol extracts of acetone-dried preparations of this tissue.

4. High levels of activity were found in ileum, lung, spleen, liver and pancreas. The activity was low in skeletal muscle, heart muscle, kidney, testis, brain and blood. The grey matter of the central nervous system contains considerably more activity than the white matter.

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The Incorporation of ^{15}N into Adenine Nucleotides and their Formation from Inosine Monophosphate by Skeletal-Muscle Preparations

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Most vertebrate skeletal muscles contain an active enzyme specific for the deamination of adenosine 5′-monophosphate (Schmidt, 1928). When skeletal muscle is repeatedly stimulated or damaged this enzyme is responsible for production of the ammonia that was the subject of detailed investigations by the Parnas and Embden schools in the 1920's [see Needham (1932) for a review of this work]. Both groups of workers considered that resynthesis of the adenosine monophosphate from inosine monophosphate occurred in muscle and Parnas (1929) in particular favoured the idea of aerobic reamination at the expense of the tissue amino acids, although he failed experimentally to demonstrate this reaction (Parnas, 1933). Subsequent studies by Kalckar & Rittenberg (1947) confirmed that reversible deamination of the 6-amino group of adenine nucleotides could occur in living muscle. These workers showed that after the administration of [^{15}N]ammonium citrate to rats, significant incorporation of the isotope could be detected in the adenosine triphosphate isolated from skeletal muscle. With systems *in vitro*, Kleinzeller (1942) was, however, unable to demonstrate in muscle homogenates the reamination either of inosine diphosphate or of inosine triphosphate by incubation with amino acids. Not surprisingly, in view of the thermodynamical considerations (Tarver & Morales, 1951), Kalckar (1947*a*) failed to demonstrate the reversibility of the 5′-adenylic deaminase reaction. More recently, however, Nekhorocheff & Wajzer (1952) and Nekhorocheff (1955) have provided evidence that, in muscle, creatine can act as a precursor of the 6-amino group of adenosine triphosphate, whereas Kometiani & Klein (1956) propose, on the basis of their experimental findings, β-alanine for this function.

The present paper is concerned with a study of the mechanism of reamination of inosine monophosphate in muscle systems. A partly purified enzyme preparation is described which catalyses the transfer of the amino group of aspartic acid to inosine monophosphate to give adenine nucleotides. Evidence is provided which indicates that the system may be similar to that described by Carter & Cohen (1955) for the synthesis of adenosine monophosphate in yeast.

A preliminary account of some aspects of this work has already been published (Newton & Perry, 1957).

METHODS

Whole-animal experiments. A total of 100 ml. of ammonium citrate solution containing 280 mg. of NH_3 nitrogen (62 atoms % excess of ^{15}N) was injected subcutaneously into adult rabbits in three equal doses at 2 hr. intervals. Animals were not fed but could move freely in their cages during the experiment. They were killed for the extraction of the muscle nucleotides 4 hr. after the final injection.

Preparation of homogenates. Back and leg muscles from a freshly killed normal rabbit were removed and chilled in 0·16 M-KCl; they were freed from adherent moisture, minced and briefly homogenized with 4 vol. of a medium so that final concentrations in the homogenate were: 0·1 M-KCl, 50 mM-glycylglycine–HCl, pH 7·5, mM-$MgCl_2$, mM-adenosine triphosphate (ATP), 10 μg. of pyridoxal phosphate/ml. and 11 mg. of $^{15}NH_4Cl$ or the equivalent amount of nitrogen as [^{15}N]aspartic acid/100 ml. The homogenate was stirred at room temperature for 30 min., the reaction was stopped by the addition of 60% (w/v) trichloroacetic acid to give a final concentration of 7% and treated at 0°

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as follows. The precipitated protein was centrifuged down, washed twice with 5% (w/v) trichloroacetic acid, and the combined supernatant fluids were extracted ten times with ether to remove the acid. The clear solution was neutralized and stored at 0°.

Homogenate fractionation. Muscle from a freshly killed animal was chilled in 0·16M-KCl, freed from adherent moisture, minced and homogenized with 4 vol. of a medium containing 0·16M-KCl and 0·05M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·4, (KCl–tris medium) in a Waring Blendor for 1 min. The soluble proteins were separated from other cell components by centrifuging at 15 000 *g* for 30 min. This fraction (soluble fraction) was used undiluted for enzyme incubations, whereas the sediment consisting of myofibrils, nuclei and some particulate material was resuspended in the KCl–tris medium to give a volume equal to that of the original homogenate.

Acetone-dried powder preparation. To the soluble fraction obtained as described above was added 2 vol. of cold acetone. The precipitated protein was washed at 0° by resuspension in acetone and centrifuging. This process was repeated once with acetone and once with ether and the dried powder was stored at −15°. Before use it was ground with ice-cold water (0·1 g./ml. of water) and dialysed overnight against distilled water at 0°. The insoluble residue was removed by centrifuging and the clear supernatant fluid used for enzyme assay.

For large-scale preparations the procedure was similar except that homogenization was carried out in 2 vol. rather than 4 vol. of KCl–tris medium.

Preparation of nucleotides from muscle extracts. The leg and back muscles were dissected from a freshly killed rabbit and immediately dropped into liquid nitrogen. While still frozen they were crushed to fine powder in a mortar, an equal volume of 10% (w/v) trichloroacetic acid was added and the suspension allowed to thaw. Liquid was separated from the precipitated protein by squeezing through muslin and the residue was again extracted with 2 vol. of 5% (w/v) trichloroacetic acid for 1 hr. at 0°. After the sediment had been removed by centrifuging, the combined supernatant liquids were clarified by filtration through cotton wool. Ten extractions with ether were carried out to remove most of the trichloroacetic acid from the aqueous phase, which was finally neutralized and stored overnight at 4°.

Separation of nucleotides. Nucleotides were separated by chromatography on columns of Dowex-1 (chloride form) by a modification of the method of Bergkvist & Deutsch (1954). The volume of resin was about 1·5 cm.³/mg. of phosphate. The resin was prepared for use by washing in sequence with methanol, 2N-NaOH and N-HCl; after the final acid washing it was washed with water until the eluate was chloride-free. The same resin was used for several experiments but was washed with NaOH and HCl between each experiment.

(i) Method (1). For complete fractionation of the adenine nucleotides the following procedure was used. (*a*) Nucleotides were placed on the column in a solution brought to pH 10·0 by the addition of aq. N-NH_3 soln. (*b*) The column was washed with water until the pH was 5·0 and the nucleotides were eluted in turn by stepwise application of the solutions (*c*)–(*h*): (*c*) 3 mN-HCl to elute adenosine monophosphate (AMP); (*d*) 5 mN-HCl to elute inosine monophosphate (IMP); (*e*) 0·01N-HCl–0·02M-NaCl to elute adenosine diphosphate (ADP); (*f*) 0·01N-HCl–0·05M-NaCl to elute inosine diphosphate (IDP); (*g*) 0·01N-HCl–0·2M-NaCl to elute ATP; (*h*) 0·1–1·0N-HCl to elute inosine triphosphate (ITP), guanosine triphosphate (GTP), etc. Fractions (25 ml.) were collected and the elution was followed by determination of the ultraviolet-absorption spectrum of the eluent in the range 220–300 mμ. Elution was continued until the extinction of the eluate was less than 0·020. At least 1 l. of eluting fluid at each concentration was used. The identities of the nucleotides in the fractions were checked by paper chromatography in the *iso*butyric acid–NH_3 solvent described by Krebs & Hems (1953). The acidic and aromatic amino acids were also retained by the resin and could be eluted with dilute acid solutions. In the later work it was necessary to avoid contamination of the ADP and ATP fractions by these amino acids and so the columns were washed with large volumes of 0·01N-HCl before these fractions were removed, i.e. in place of (*c*) and (*d*) but before (*e*).

(ii) Method (2). When nucleotide solutions containing only a few components were to be analysed, and complete separation of the adenine nucleotides was not required, the following procedure was used. (*a*) Adsorption of the nucleotides at pH 10·0 as before. (*b*) Washing with 2 l. of water. (*c*) Elution with 1 l. each of solutions of increasing acidity: 1, 2, 3, ..., 9, 10 mN-HCl. Samples of 1 l. were collected, the u.v. absorption at 248 and 260 mμ was determined and the fractions were discarded. This washing removed AMP, glutamic acid, aspartic acid and IMP. (*d*) Elution with 0·1N-HCl to remove ADP and ATP. This eluate was collected in 5 ml. fractions and the nucleotide content determined as before.

Nucleotide solutions prepared by either method were neutralized and if the volume was large they were concentrated by freeze-drying, or by boiling if the sample was to be analysed immediately for ^{15}N content. In pure fractions the nucleotide concentrations were estimated from their absorption in the ultraviolet with the extinctions determined by Kalckar (1947*b*).

Preparation of adenine from adenosine triphosphate. The ATP was hydrolysed by boiling in N-HCl for 1 hr. The adenine was precipitated as the silver salt, dissolved in 0·5N-HCl and recrystallized from the neutralized solution after removal of the precipitated AgCl and concentration to small volume in the water bath at 100°. After a further recrystallization from water the adenine moved as a single spot, identical with that obtained with a known sample of adenine, in the propan-2-ol–conc. HCl–water (68:17·6:14·4) system of Wyatt (1951). The preparation was negative to the ninhydrin test for amino acids.

Preparation of adenosine mono- from adenosine triphosphate. [^{15}N]ATP, isolated by elution from a Dowex-1 column by 0·1N-HCl as previously described, was used for the preparation of [^{15}N]AMP. The HCl concentration was reduced to about mM by the addition of Ag_2CO_3 and the neutralized solution of ATP was then hydrolysed to AMP by a method suggested by Dr M. Clark, which involved heating in 50% (v/v) pyridine at 100° for 6 hr. Aqueous pyridine was removed by distillation *in vacuo*, and the dry residue was taken up in water and again dried *in vacuo*. The nucleotide was then dissolved in water and separated from unhydrolysed ATP and ADP (about 10% of the total nucleotide) by chromatography on Dowex-1. After neutralization of the eluate containing the AMP it was

concentrated by freeze-drying. The AMP ran as one spot in the *iso*butyric acid–NH_3 system of Krebs & Hems (1953).

Liberation of the 6-amino nitrogen of adenosine mono- and tri-phosphate. AMP prepared as described above was deaminated by means of the specific 5′-AMP deaminase of rabbit myosin, by incubating together for 1 hr. at 30°: 1 ml. of myosin (2·7 mg. of protein/ml.) prepared as described by Perry (1955) and dissolved in 0·5 M-KCl, 0·5 ml. of M-succinate–NaOH buffer, pH 6·0, and 5 ml. of 14 mM-AMP. In some cases equivalent amounts of ATP itself were deaminated by incubation with 1 ml. of a 2% suspension of myofibrils, prepared as described by Perry & Grey (1956), in 0·1 M-KCl, 0·1 M-tris–HCl, pH 7·4, and 10 mM-$MgCl_2$, in a total volume of 5 ml. Prior conversion into AMP was not necessary in this case because of the adenosine triphosphatase and myokinase present in the myofibrils together with the 5′-adenylic deaminase. After being deproteinized with 1 ml. of 60% trichloroacetic acid, the solution was made alkaline by the addition of 10 ml. of M-$NaHCO_3$–Na_2CO_3 buffer, pH 10·0. The NH_3 was distilled into 50 mM-H_2SO_4 in the apparatus described by Sprinson & Rittenberg (1948). The NH_3-free solution remaining was digested with H_2SO_4 for the determination of the ^{15}N content of the IMP.

^{15}N-labelled ammonium salts. An aqueous solution of $^{15}NH_4NO_3$ supplied by the Eastman Kodak Co. (62 atoms % excess of ^{15}N in the ammonium group) was made alkaline and converted into citrate or chloride by distillation of the NH_3 evolved into a solution of the appropriate acid. For injection into animals labelled ammonium citrate was prepared from 1·8 g. of NH_4NO_3, the solution was neutralized and the volume adjusted to 100 ml. It was sterilized by filtration through a Seitz filter and stored at 1–2° until used.

L-[^{15}N]*Aspartic acid.* This was prepared by the method described by Wu & Rittenberg (1949), with the following modification. Before the final recrystallization of the aspartic acid the solution was freed from contamination with copper salts by shaking with a solution of 8-hydroxyquinoline in $CHCl_3$. When the [^{15}N]aspartic acid was mixed with known aspartic acid it gave a single spot on chromatography in pyridine–acetic acid–water (50:35:15, by vol.) (Decker & Riffart, 1950) and in butan-2-ol–formic acid–water (70:10:20, by vol.) followed by phenol–water–conc. aq. NH_3 soln., sp.gr. 0·88 (80:20:0·3, by vol.). Electrophoresis on paper buffered with pyridine–acetic acid–water (1:10:89, by vol.), pH 3·5, for 1 hr. at 40 v under toluene (Sanger, personal communication) also gave a single spot. After degradation with H_2SO_4 the aspartic acid gave 95% of the theoretical nitrogen. Atoms % excess of ^{15}N, 59·2.

L-[^{15}N]*Glutamic acid.* This amino acid was prepared from the hydrolysed protein of *Escherichia coli* grown in a medium containing $^{15}NH_4Cl$ as the only nitrogen source. The *E. coli* were grown in 1 l. of the C medium described by Roberts, Abelson, Cowie, Bolton & Britten (1955) containing 1 g. of $^{15}NH_4Cl$ (62 atoms % excess of ^{15}N) and 10 g. of glucose as energy source. After hydrolysis of the protein with 6 N-HCl for 24 hr. at 100° under reflux the barium salts of the aspartic acid and glutamic acid were precipitated as described by Jones & Moeller (1928). These salts were freed from barium by solution in 0·1 N-H_2SO_4 and the amino acids were separated by ion-exchange chromatography with Dowex-50 (HCl form) by a method described by Mr M. Rees (personal communication). Fractions containing glutamic acid only were concentrated to a syrup from which crystals of glutamic acid–HCl were obtained. These were washed in ethanol and ether and dissolved in water.

The glutamic acid was chromatographically pure in a pyridine–acetic acid–water (50:35:15, by vol.) solvent system (Decker & Riffart, 1950), and on zone electrophoresis at pH 3·5 no trace of contamination by aspartic acid could be detected. Atoms % excess of ^{15}N, 54.

Preparation of samples for ^{15}N analysis. Solutions containing about 1 mg. of nitrogen were boiled in a 50 ml. Kjeldahl flask with 5 ml. of M-$NaHCO_3$–Na_2CO_3 buffer, pH 10·0, for 30 min. to remove contaminating NH_3. Tests showed that this process removed quantitatively NH_3 added to nucleotide solutions without affecting the 6-amino group of the adenine ring. Digestion was carried out overnight with 2 ml. of 98% (w/w) H_2SO_4 and 0·3 ml. of the $HgSO_4$ catalyst recommended by Hiller, Plazin & Van Slyke (1949), and the NH_3 was distilled in the modified distillation apparatus described by Sprinson & Rittenberg (1948), into a solution of 50 mM-H_2SO_4. After removal of a sample for estimation of the NH_3 by nesslerization (Johnson, 1941), the acid distillate was concentrated by boiling to about 2 ml. and finally stored in a sealed tube for analysis.

Controls containing unlabelled NH_4Cl were digested with each experimental series and their ^{15}N content was determined. This was always between 0·37 and 0·39 atom % ^{15}N, and was the figure used as 'normal' ^{15}N content.

Analysis of ^{15}N content. The samples were converted into nitrogen by the method of Sprinson & Rittenberg (1949) and the ^{15}N content was determined on a 180° mass spectrophotometer in this Department. A few samples were also analysed by Mr G. Dickinson at the National Institute for Medical Research, Mill Hill, and the values obtained agreed very well with those obtained with the Cambridge Instrument. The accuracy of the determinations was such that a standard error of $\pm 0{\cdot}005$ atom % ^{15}N was obtained.

RESULTS

A number of preliminary experiments were carried out in which IMP was incubated with various muscle preparations and nitrogen sources such as NH_4Cl, glutamic acid and aspartic acid. Aspartic acid in particular was selected in view of the similarities between the system under investigation and the amination of citrulline to give arginine (Ratner, 1947). Its likely role as precursor was further suggested by the results of AMP synthesis in yeast (Carter & Cohen, 1955), which were published during the course of this investigation. Incubations were carried out in the presence of active aerobic and anaerobic phosphorylating systems such as pigeon breast-muscle mitochondria isolated by the method of Chappel & Perry (1954) and rabbit muscle homogenates and acetone-dried powder extracts. Under these conditions it was hoped that any AMP formed from IMP would be trapped by phosphorylation to ADP or ATP, which nucleotides are not deaminated by the active 5′-adenylic deaminase present in the preparations.

With α-oxoglutarate as oxidizable substrate and the nitrogen sources mentioned above, IMP could not replace AMP as a phosphate acceptor for oxidative phosphorylation with pigeon breast-muscle mitochondria.

With the anaerobic systems and fructose diphosphate as substrate for glycolysis it was not possible to show appreciable uptake of inorganic phosphate with IMP as acceptor. There were indications that some AMP formation had occurred, however, for at times an increase in ATP level after incubation could be detected by paper chromatography.

Incorporation of ^{15}N into adenosine triphosphate in vivo

Rabbit muscle offers certain advantages for the study of the incorporation of ^{15}N into ATP and it was first necessary to confirm that the findings of Kalckar & Rittenberg (1947) with the rat were applicable to the rabbit. For this purpose [^{15}N]-ammonium citrate was injected into adult rabbits and the trichloroacetic acid extract of their skeletal muscles was chromatographed on a Dowex-1 column. Fig. 1 illustrates a typical elution chromatogram

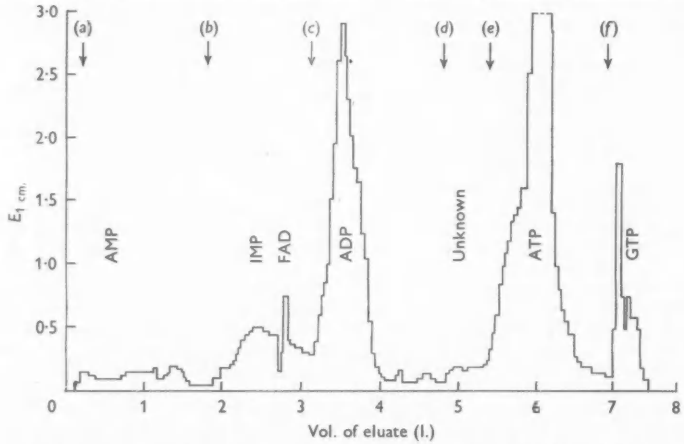

Fig. 1. Chromatography of nucleotide fraction isolated from rabbit skeletal muscle after injection of [^{15}N]ammonium citrate; Dowex-1, method 1. Stepwise elution: (*a*) 3 mM-HCl; (*b*) 10 mM-HCl; (*c*) 10 mM-HCl–20 mM-NaCl; (*d*) 10 mM-HCl–50 mM-NaCl; (*e*) 10 mM-HCl–0·2 M-NaCl; (*f*) M-HCl. Maximum reading of ATP peak, $E_{1\ cm}$ 34·5.

Table 1. *Distribution of ^{15}N in nucleotide fractions isolated from rabbit skeletal muscle after injection of [^{15}N]ammonium citrate*

Fractionation was carried out by method 1 (see Methods section). FAD, Flavinadenine dinucleotide; ITP, inosine triphosphate; GTP, guanosine triphosphate.

	Fraction no.	Identified compounds	Nucleotide N (% of total N)	Atoms % excess of ^{15}N
Expt. 1	1	AMP + glutamic acid + aspartic acid	22·5	0·091
	2	ADP + IMP + FAD	25	0·058
	3	Unknown	93*	0·512
	4	ATP	101	0·091
	5	ITP + GTP	92	0·072
		ATP amino group	—	0·153
Expt. 2	1	AMP + aspartic acid + glutamic acid	7	0·043
		AMP	8	0·027
	2	ADP + IMP + FAD	24	0·023
		ADP	102	0·023
	3	Unknown	100*	0·404
		Unknown + ATP	100*	0·097
	4	ATP	110	0·036
	5	ITP + GTP etc.	90	—

* Extinction of the unknown was assumed to be identical with that of ATP.

Table 2. *Incorporation of* ^{15}N *into adenine nucleotides by incubation of muscle fractions with labelled aspartic acid*

Incubations were carried out at 20° for 30 min. in 0·1 M-KCl, 10 mM-Sorensen phosphate buffer, pH 7·4, 2 mM-IMP, mM-AMP, mM-$MgCl_2$, 2 mM-fructose 1:6-diphosphate, 0·002% of coenzyme I, 10 μg. of pyridoxal phosphate/ml., 2 mM-[^{15}N]-aspartic acid. The enzyme fraction represented 20% of the total volume except in the acetone-dried powder experiment, when, in 22 ml. total volume, 20 ml. consisted of enzyme extract.

	Enzyme preparation	IMP (μmoles)	ADP + ATP (μmoles)	Atoms % excess of ^{15}N in ADP + ATP	Activity*
Expt. 1	Whole homogenate	268	180	2·04	7·45
	Particulate	400	46	0·91	0·83
	Soluble	275	275	1·51	8·4
Expt. 2	Fresh soluble fraction	70	30	0·73	4·6
	Dialysed soluble fraction	37	11	0·90	2·68
	Aqueous extract of acetone-dried powder	39·5	16	0·36	1·08

* Activity is expressed as μg.atoms of ^{15}N incorporated into ADP + ATP/g. wet wt. of original muscle.

obtained under these conditions. From analysis of the fractions by spectrophotometry and paper chromatography it was clear that dicarboxylic amino acids were present in the fraction containing AMP and that IMP and flavin nucleotides were associated with the ADP. No amino acids could be detected in fractions eluted after the AMP.

Nucleotide fractions prepared in this way were analysed for their ^{15}N content. Table 1 contains the results of two experiments, in one of which the main fractions were further subdivided to improve the purity of the nucleotide samples. It can be seen from the results that ^{15}N was present in all the fractions containing adenine nucleotides. Although AMP could not be obtained free from other nitrogen-containing compounds, all the nitrogen could be accounted for by ADP and ATP in their respective fractions and both were significantly labelled with ^{15}N. The specific activity of these compounds was in both cases much higher than that obtained by Kalckar & Rittenberg (1947) for the adenosine polyphosphates isolated from rat muscle. A precise comparison between the degree of labelling of the various adenosine phosphates was not possible owing to the presence of contaminating substances particularly in the AMP fraction.

Incorporation of ^{15}N *into adenosine triphosphate* in vitro

Whole-muscle homogenates. When fresh rabbit muscle was homogenized at 0° in the appropriate medium (see Methods section) containing [^{15}N]-aspartic acid, and subsequently incubated at 30°, appreciable incorporation of ^{15}N could be demonstrated in the ATP fraction isolated from the deproteinized extract.

The incorporation achieved, however, with $^{15}NH_4Cl$ alone as the source of nitrogen was barely significant, but the degree of labelling could be increased slightly by the addition of unlabelled aspartic acid and glutamic acid to the incubation mixture. When aspartic acid replaced the NH_4Cl as the source of ^{15}N, the ATP recovered was highly labelled, e.g. 0·575 atom % excess, the content of ^{15}N being at least ten times that observed *in vivo* with $^{15}NH_4Cl$.

To confirm that the high content of ^{15}N found in the ATP fraction was not due to contamination with other compounds adenine was prepared from the fraction. On analysis this was shown to have a ^{15}N content only slightly less than that of the ATP fraction from which it had been prepared.

Fractionation of muscle homogenates. As a preliminary to purification of the enzyme system responsible for the incorporation of ^{15}N into ATP, rabbit-muscle homogenates prepared in the KCl–tris medium were separated into a so-called soluble fraction and the sediment obtained by centrifuging at 15 000 *g* for 20 min. These two fractions were then incubated independently with [^{15}N]aspartic acid, ATP, IMP and fructose 1:6-diphosphate, and the ^{15}N content of the ATP present in the medium after incubation was then determined as before. Both the soluble and the sedimented fractions gave rise to ATP of high ^{15}N content (Table 2). The breakdown of ATP due to dephosphorylation and deamination by the myofibrils in the sedimented fraction, however, was extensive, with the result that the yield of labelled ATP in this system was low. The total amount of ^{15}N recovered in the ATP isolated after incubation with the soluble fraction was 10 times greater than that recovered under similar circumstances with the sedimented fraction.

Table 2 includes also the results of experiments to show the effects of various treatments on the ability of the soluble fraction to incorporate ^{15}N from labelled aspartic acid into ATP. Both dialysis and precipitation as the acetone-dried powder led to some loss in activity. Considerable activity survived the latter treatment, however, and the acetone-dried powder provided a convenient source of the enzyme for subsequent studies.

Localization of ^{15}N in adenosine triphosphate

When ATP isolated from muscle, after administration *in vivo* of labelled ammonium citrate, was incubated with myofibrils, analysis of the isotope content of the NH_3 liberated showed that the ^{15}N concentration in the 6-amino group was about twice that in the molecule as a whole. This indicated that although there was greater specific incorporation into the 6-amino group than into the whole purine, some isotope had also been introduced into the adenine ring, presumably as a consequence of fresh synthesis occurring during the course of the experiment (Table 3). When muscle homogenates were used with [^{15}N]aspartic acid as the nitrogen source the incorporation was confined practically entirely to the 6-amino group of the ATP. The results obtained in Expt. 2, Table 3, show that enzymic deamination of the AMP, obtained by degradation of ATP isolated from muscle homogenates after incubation, produced NH_3 4·9 times as highly labelled as the original AMP. Only about 2% of the total ^{15}N was present in the IMP left after deamination.

Properties of the acetone-dried powder extract

Acetone-dried powders prepared from rabbit muscle were not as active per gram of original tissue as fresh homogenates, but they possessed the advantage of retaining activity after storage at −15° for periods up to 3 weeks. In addition, study of the reamination of IMP was facilitated with aqueous extracts of these powders because on a nitrogen basis the 5′-adenylic deaminase activity was only about 5% of that of whole-muscle homogenates; the adenosine triphosphatase activity was also much less. Usually acetone-dried powders were obtained from rabbit skeletal muscle but an active preparation was also made from pigeon breast muscle. An extract from the acetone-dried powder of rabbit heart gave a relatively small incorporation of isotope into ATP.

In many of the experiments with homogenates and extracts of acetone-dried powder, fructose 1:6-diphosphate and diphosphopyridine nucleotide were added to promote the phosphorylation by anaerobic glycolysis of any AMP formed. Results of an experiment designed to show the effect of omitting various components from the usual incubation system are presented in Table 4. Omission of fructose 1:6-diphosphate reduced the extent of incorporation only by a minor degree, and added ATP was likewise apparently not essential for the system. In fact the greatest incorporation obtained in this type of experiment, both from the point of specific activity and total incorporation,

Table 3. *Concentration of ^{15}N in the 6-amino group of adenosine triphosphate*

Incubation medium for the experiment *in vitro* contained 5 mM-IMP, 5 mM-ATP, mM-$MgCl_2$, 2 mM-[^{15}N]aspartic acid, 10 μg. of pyridoxal phosphate/ml., 0·1 M-KCl, 50 mM-Sorensen phosphate buffer, pH 7·4. Enzyme extract was 20% of total volume.

Conditions	Nucleotide fraction or derivative	Atoms % excess of ^{15}N
Expt. 1		
[^{15}N]Ammonium citrate injected *in vivo*	ATP*	0·091
	NH_3 (from ATP 6-NH_2 group)	0·153
Expt. 2		
[^{15}N]Aspartic acid incubated with soluble fraction of muscle homogenate	AMP (from ATP)	0·278
	NH_3 (from AMP 6-NH_2 group)	1·350
	IMP (from AMP)	0·028

* Total N of this fraction was 3930 μg.atoms. From u.v.-absorption data it was estimated that 4000 μg.atoms of N were present as adenine nucleotide.

Table 4. *Adenine and inosine nucleotides and the incorporation of ^{15}N into adenosine polyphosphates from aspartic acid by acetone-dried powder extracts*

Incubation was for 1 hr. at 20° in 50 mM-Sorensen phosphate buffer, pH 7·4, 5 mM-$MgCl_2$, 0·002% of coenzyme I, 5 mM-[^{15}N]aspartic acid and, where added, 7 mM-IMP, 5 mM-ATP, 5 mM-AMP and 10 mM-fructose 1:6-diphosphate (FDP). Total volume, 10 ml. (including 5 ml. of acetone-dried powder extract).

Additions to basic medium	IMP (μmoles)	ADP + ATP (μmoles)	^{15}N in ATP (atoms % excess)	^{15}N incorporated (% of total)
AMP, IMP, FDP	69	49	0·293	2·4
AMP, IMP, FDP boiled enzyme	71	47	Nil	Nil
ATP, IMP	69	49	0·253	2·11
IMP, FDP	59	9	1·90	2·89
AMP, FDP	0	44	0·221	1·65
ATP	0	45	0·120	0·91

was obtained when IMP and fructose 1:6-diphosphate alone were added to the medium. The net synthesis of ATP in this system was, however, small. The complete system was heat-labile and the extent of incorporation rose with time. After 4 hr. 28·7% of the aspartic acid ^{15}N had been incorporated into the adenosine polyphosphate fraction, which had increased in amount with a complementary decrease in the IMP level compared with the concentrations of nucleotides at the beginning of the incubation (Table 5).

Precursors for the nitrogen of the 6-amino group of adenosine triphosphate

The most effective of the three possible precursors for the 6-amino group of the adenine nucleotides which were tested was aspartic acid. This applied both with homogenates and with extracts of acetone-dried powders of rabbit muscle. In the latter system there was no incorporation of ^{15}N from $^{15}NH_4Cl$ (Table 6) and the results obtained with this substance in the whole animal were apparently due to prior conversion in the animal's tissues of the inorganic nitrogen into some other substance which could act as a direct precursor.

Nekhorocheff (1955) has reported that creatine and arginine can act as nitrogen donors for the reamination of IMP. As dialysed acetone-dried powder extracts could catalyse the reamination of IMP in the absence of added creatine or arginine it seems unlikely that these substances were acting as precursors in the studies reported here. Creatine could not be detected in extracts of the acetone-dried powders and the arginine content was always less than 5 μg./ml.

When [^{15}N]glutamic acid was used as the source of nitrogen, some slight but significant incorporation was observed. It was possible that in this system the glutamic acid was acting by transamination reactions to produce labelled aspartic acid from traces of oxaloacetate present in the extract. This last-named reaction did occur in the extracts, for incubation of the enzyme with glutamate alone produced traces of chromatographically identifiable

Table 5. *Time course of transfer of ^{15}N from aspartic acid into adenosine polyphosphates by an acetone-dried powder extract of muscle*

Incubation was carried out at 20° in 10 mM-Sorensen phosphate buffer, pH 7·4, mM-$MgCl_2$, 0·002% of coenzyme I, 5 mM-[^{15}N]aspartic acid, 5 mM-IMP, 5 mM-AMP, 10 mM-fructose 1:6-diphosphate, 10 μg. of pyridoxal phosphate/ml. Total volume, 5 ml. (including 2·5 ml. of aqueous acetone-dried powder extract).

Incubation time (hr.)	IMP (μmoles)	ADP + ATP (μmoles)	^{15}N in ADP + ATP (atoms % excess)	Total incorporation (% of total ^{15}N)
0	22·2	(23·0)*	—	—
0·5	16·5	28	0·394	3·0
1·0	15·5	29	0·770	7·3
2·0	10·0	36	1·018	12·4
4·0	3·6	41	2·071	28·7

* Measured as AMP.

Table 6. *Glutamic acid, aspartic acid and ammonium chloride as source of ^{15}N incorporated into adenine nucleotides*

Incubations were carried out at 20°. Basic medium contained 5 mM-ATP, 5 mM-IMP, 10 mM-fructose 1:6-diphosphate, 10 mM-Sorensen phosphate buffer, pH 7·4, mM-$MgCl_2$ and 0·002% of coenzyme I. To this were added 5 mM-oxaloacetate or α-oxoglutarate, 5 mM-[^{15}N]-aspartate, -glutamate or $^{15}NH_4Cl$ as indicated. Total volume, 6 ml. (including 3 ml. of acetone-dried powder extract), except in Expt. 3 where 2·5 ml. of extract was used and the total volume was 5 ml.

Expt. no.	Source of N	Incubation time (hr.)	IMP (μmoles)	ADP + ATP (μmoles)	^{15}N incorporated into ADP + ATP (atom % excess)
1	Aspartic acid	4	30	32·5	0·270
	NH_4Cl	4	26	35	Nil
2	Aspartic acid	4	30	33	0·260
	Aspartic acid + oxaloacetic acid	4	—	38	0·270
	Glutamic acid	4	32	30	0·003–0·013
	Glutamic acid + oxaloacetic acid	4	32	31	0·061
3	Aspartic acid	1	19	27	0·87
	Aspartic acid + α-oxoglutarate	1	22	25	0·447

aspartic acid. Further suggestions that glutamate was not acting directly as a nitrogen source, but rather through transamination reactions, were obtained by the following observations. With glutamate as the source of ^{15}N addition of oxaloacetate increased the incorporation of isotope into the adenosine polyphosphates, whereas with aspartate as the source of the isotope, incorporation decreased when α-oxoglutarate was added to the system.

Evidence for the existence of an intermediate in the reamination of inosine monophosphate by aspartic acid

It was first observed that a small fraction, not identical with any of the known inosine or adenine nucleotides, could be isolated by chromatography of the nucleotide extract from whole muscle obtained after the injection of [^{15}N]ammonium citrate. This small fraction (Table 1, fraction 3), which was eluted after ADP, consistently possessed much higher concentrations (usually greater than tenfold) of ^{15}N than any of the fractions containing the known adenine nucleotides. It had an absorption spectrum in the ultraviolet region with a maximum at 265 mμ at pH 2·0. On analysis it was shown to possess the following characteristics. Hydrolysis in N-HCl at 100° for 1 hr. gave rise to adenine, which was identified chromatographically, and another compound which exhibited maximum absorption in the ultraviolet at 273 mμ. Attempts to characterize fraction 3 further were unsuccessful, due partly to the high salt content and partly to the small quantities available.

A similar compound was also found to accumulate after incubation of acetone-dried powder extracts with [^{15}N]aspartic acid, IMP and fructose 1:6-diphosphate. This compound was present in the combined ADP and ATP fraction (i.e. that eluted between 0·01 N- and 0·1 N-HCl). For example, in the experiment illustrated in Table 5 it represented 20% of the combined ADP+ATP fraction. Some acetone-dried powder extracts which possessed low ability to incorporate ^{15}N into ATP still promoted the formation of this substance, which was often more easily detected in such systems than in those rapidly catalysing incorporation of ^{15}N into ATP. The substance was not separated from IMP in the *iso*butyric acid–NH_3 system (Krebs & Hems, 1953), but satisfactory resolution was achieved in a system containing acetic acid–water–acetone (1:4:10, by vol.) and its formation could be semi-quantitatively followed under these conditions. The unidentified compound moved in front of IMP and the adenine nucleotides in this system. Elution of the unidentified spot from the chromatogram provided enough to measure the ultraviolet-absorption spectrum. It was characterized by a maximum at 265–267 mμ in 0·1 N-HCl which shifted to 270 mμ at pH 13·0 (Fig. 2). Hydrolysis for 30 min. in N-HCl at 100° shifted the absorption maximum to 275 mμ.

In the usual incubation medium containing 10 mM-IMP, 10 mM-aspartic acid and 10 mM-fructose 1:6-diphosphate the new spot was readily detectable. Fructose 1:6-diphosphate was not an essential addition for its formation but in the absence of either IMP or aspartic acid it could not be detected on the chromatogram. Replacement of aspartic acid by glutamic acid also failed to produce a detectable spot.

On prolonged incubation of IMP with aspartic acid in the presence of the aqueous extract of acetone-dried powder of muscle the characteristic inosine nucleotide ultraviolet-absorption spectrum with a maximum at 248 mμ shifted to one with the maximum in the range 258–265 mμ. A shift to the longer wavelength would be expected on formation of adenine compounds. This change in absorption spectrum suggested the possibility of following the reaction in semi-purified systems by a spectrophotometric method. The wavelength 280 mμ was chosen as convenient for this purpose and a regular although small increase in absorption at 280 mμ occurred as the reaction progressed. This increase did not occur in the absence of aspartic acid or IMP, and glutamic acid was not effective in replacing aspartic acid.

The experiments indicated that both IMP and aspartic acid were essential for the formation of an

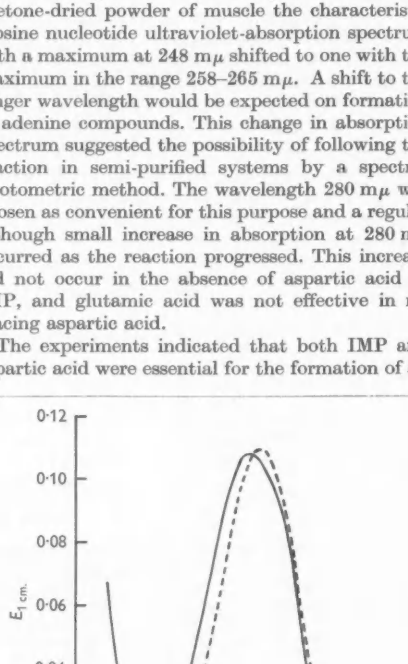

Fig. 2. Ultraviolet-absorption spectra of unidentified compound formed on incubation of IMP and aspartic acid with an aqueous extract of acetone-dried powder of muscle. —, At pH 1·0 (0·1 M-HCl), max. 265–267 mμ; - - - -, at pH 13·0 (0·1 M-NaOH), max. 268–270 mμ.

Table 7. *Incorporation of ^{15}N into unidentified compound*

Incubation was for 4 hr. at 20° in medium identical with the basic medium described in Table 6. Total volume, 10 ml. (including 5 ml. of acetone-dried powder extract).

Nucleotide	Amount (μmoles)	Concn. of ^{15}N (atoms % excess)
IMP	26	—
ADP	1·1	1·320
Unknown	1·8	9·20
ATP	17	0·186

unidentified compound, which was probably an intermediate in the amination of IMP by this amino acid. In one case it was possible to isolate enough of the compound to carry out ^{15}N determinations (Table 7). The ^{15}N content was high, approximately one-sixth of that of the original aspartic acid, whereas the isotope level in the adenine nucleotides was much lower. This level of labelling of the unknown is close to the value which would be expected if it were adenylosuccinate. In this experiment, as in others carried out with systems *in vitro*, the ADP was more highly labelled than the ATP, which is consistent with the hypothesis that IMP is first reaminated to AMP which is subsequently phosphorylated in turn to ADP and ATP.

The properties of the intermediate isolated both in the experiments *in vivo* and *in vitro* are very similar to those of adenylosuccinate (Carter & Cohen, 1955), which was first shown to be an intermediate in the formation of AMP from IMP in yeast preparations. This compound can also be produced in yeast preparations by the incubation of fumarate and AMP. With muscle preparations, however, it was not possible to detect chromatographically the formation of the unidentified compound on incubation of fumarate and AMP.

DISCUSSION

In view of the findings reported above with the rabbit and pigeon and those of Kalckar & Rittenberg (1947) with the rat the active metabolism of the 6-amino group of adenine nucleotides is apparently a widespread reaction in skeletal muscle. Experiments with the aqueous extracts of acetone-dried powders indicate that in such systems the amino group of aspartic acid is an active precursor of the purine amino group whereas ammonium chloride is ineffective. The slight activity of glutamic acid is probably due to the transfer of its amino group to aspartic acid by transamination systems rather than to glutamic acid itself acting as a direct precursor.

As aspartic acid was the only nitrogen source which needed to be added to dialysed enzyme preparations it seems unlikely that either creatine (Nekhorocheff & Wajzer, 1952; Nekhorocheff, 1955) or β-alanine (Kometiani & Klein, 1956) play a role in the reamination process studied here. The possibility cannot be excluded, however, that aspartic acid is decarboxylated to β-alanine by muscle preparations by some reaction not yet described. It is perhaps more likely that the results with β-alanine are explained by transfer of its amino group to aspartic acid by transamination reactions. Certainly in the yeast system for AMP formation from IMP (Carter & Cohen, 1955) it has been clearly shown that aspartic acid donates the amino group with the formation of adenylosuccinate as an intermediate. The widespread nature of this reaction is suggested from evidence which is accumulating with other systems (Abrams & Bentley, 1955; Liebermann, 1956). Significantly, in the rabbit-muscle preparations an adenine-containing compound highly labelled with ^{15}N and presumably an intermediate was formed. This compound contained 9·2 atoms % excess of ^{15}N, which is close to the expected value of 11·8 atoms % excess if it were adenylosuccinate. Also the limited study of the properties of the unknown compound which have been carried out are completely compatible with it being adenylosuccinate. Davey (1959) has published preliminary findings confirming the original indications (Newton, 1957; Newton & Perry, 1957) that adenylosuccinate is formed in the acetone-dried powder system we have used. In the work of Davey (1959) no details of the identification of adenylosuccinate are given other than the ultraviolet spectral change on incubation of IMP and aspartate in the appropriate system.

So far as one can conclude from these studies, the capacity of the muscle for reaminating IMP is much less than its potential AMP-deaminating ability. Indeed unless the 5′-adenylic deaminase activity of preparations was reduced in some way, e.g. by making acetone-dried powder, it was difficult to demonstrate an appreciable net formation of adenine nucleotides even when all requirements for the reaction were provided.

The extent to which deamination of AMP accompanies muscle contraction is far from clear. Reports that 5′-adenylic deaminase cannot be detected in certain muscles (Gilmour & Calaby, 1953; Anina, 1955) suggest that deamination need not accompany contraction. Experiments on whole muscle are more equivocal, for analysis of the chemical changes accompanying the rising phase of a single twitch in tortoise muscle has produced no evidence of IMP production (Mommaerts, 1955), although some difficulties in its detection are encountered in this muscle. Nevertheless Lange

(1954–55) could demonstrate an increase in IMP production during short periods of activity in frog rectus abdominus.

More recently by the analysis of changes in the ultraviolet absorption of light transmitted through a muscle fibre it has been concluded that a conversion of adenine into inosine compounds occurs during a single twitch (Wajzer, Weber, Lerique & Nekhorocheff, 1956). Although there is no doubt that IMP is formed in most skeletal muscles the precise stage in the cycle of chemical events when this occurs has yet to be determined. Nevertheless, when the nucleotide is produced it can be reconverted into AMP at the expense of amino acid nitrogen by enzyme systems endogenous to the muscle.

SUMMARY

1. The distribution of ^{15}N in the nucleotides of rabbit skeletal muscle after the administration of [^{15}N]ammonium citrate has been investigated.

2. Incubation of inosine monophosphate with [^{15}N]aspartic acid in the presence of homogenates or extracts of acetone-dried powders made from skeletal muscle produced labelled adenine nucleotides. In these systems no amination of inosine monophosphate took place with ammonium chloride as the nitrogen source and glutamic acid was only very slightly effective.

3. With muscle extracts practically all the isotope incorporated into the adenine nucleotides was localized in the 6-amino group. Nitrogen of both the purine ring and the 6-amino group of muscle adenine nucleotides was labelled after injection of [^{15}N]ammonium citrate.

4. Some properties of an aqueous extract of acetone-dried powder of muscle catalysing amination of inosine monophosphate by aspartic acid have been studied.

5. A compound, highly labelled with ^{15}N, has been isolated from whole muscle and from muscle extracts after incubation with inosine monophosphate and aspartic acid. Those properties of this substance which have been studied are identical with those of adenylosuccinate.

Our thanks are due to Dr G. H. Smith and Mr B. Boon of this Department for their help in carrying out a large number of ^{15}N estimations. We are also grateful to Mr G. Dickinson of the National Institute for Medical Research, who carried out similar determinations for us on several occasions. The cost of this investigation was in part defrayed by a research-expenses grant (to S. V. P.) from the Medical Research Council.

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The Lipids of Whole Blood

2. THE EXCHANGE OF LIPIDS BETWEEN THE CELLULAR CONSTITUENTS AND THE LIPOPROTEINS OF HUMAN BLOOD*

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In Part 1 (James, Lovelock & Webb, 1959) and in the earlier work of Altman, Watman & Solomon (1952), it was reported that the cellular constituents of the blood are able to incorporate activity from sodium [*Me*-^{14}C]acetate into their lipid components. It was also shown that the radioactive lipids synthesized by the cells are transferred during the course of incubation *in vitro* to the plasma lipoproteins.

From this earlier work little or no information is available on the contribution of the cells to the lipid metabolism of the blood, or indeed to that of the whole animal. This paper reports experiments designed to determine quantitatively the turnover of the major lipid components, namely the phospholipids, the neutral fat and the unsaponifiable lipids, between the cells and the lipoproteins of the plasma.

METHODS

Incubation of blood. During the incubation sterile procedures were used throughout. Freshly drawn whole blood (100 ml.) was added to a mixture (15 ml.) of citric acid–sodium citrate–dextrose, penicillin, streptomycin sulphate (100 units each/ml. of whole blood) and ethylenediaminetetra-acetic acid (0·05 ml. of 0·1% solution). Sodium [*Me*-^{14}C]acetate (1 μc/ml.; specific activity 13·6 μc/mg.) was added. The mixture was incubated at 34·5° with gentle agitation in a roller-tube apparatus.

Separation of cells and fractionation of lipoproteins. At different time intervals, 10 ml. samples were withdrawn from the incubation mixture and centrifuged for 5 min. The cells were washed once with 0·9% NaCl (3 ml.) and then added in a thin stream to 100 ml. of ether–ethanol (1:2, v/v). To the plasma and 0·9% NaCl washings dextran sulphate (0·5 ml. of 1% solution) was added and mixed. After 30 min. the precipitated β-lipoprotein–dextran sulphate complex was removed by centrifuging (Oncley, Walton & Cornwell, 1957) and transferred to ether–ethanol. The supernatant liquid which contained the α-lipoprotein was added slowly to ether–ethanol.

Fractionation of the lipids. The protein precipitated by ether–ethanol was removed by filtration and the filtrate concentrated to dryness *in vacuo* at low temperature. Where necessary the last traces of water were removed by the repeated addition of ethanol, followed by distillation. The residue was obtained as a thin film inside the flask. This was immediately extracted twice with 1 ml. of acetone (extract A) and the residual acetone removed by warming *in vacuo*. Phosphorus analyses indicated that extract A contained negligible quantities of phospholipid.

Hydrolysis of phospholipids. The residue from acetone extraction, dissolved in a mixture of aqueous 50% (v/v) ethanol (5 ml.) and aqueous 40% (w/v) KOH (1 ml.), was heated at 60° for 4 hr. After the mixture had cooled to room temperature, water (3 ml.), acetic acid (4 drops) and 10N-H_2SO_4 (2 ml.) were added and mixed. When cool the mixture was extracted with light petroleum (40–60°; 4 ml., 1 ml. and 1 ml.).

Hydrolysis of neutral fat. The acetone extract (A) was concentrated to dryness under reduced pressure and hydrolysed for 4 hr. at 60° in a mixture of ethanol (5 ml.) and aqueous 40% (w/v) KOH (1 ml.). Water (3 ml.) and acetic acid (4 drops) were added and the hydrolysate was extracted three times with light petroleum (4 ml., 1 ml. and 1 ml.) to remove unsaponifiable fat. Sulphuric acid (10N; 2 ml.) was added and the mixture again extracted with light petroleum as before to obtain the saponified fatty acids.

After the hydrolyses of lipids the unmetabolized sodium [*Me*-^{14}C]acetate remained substantially in the aqueous layer during the extraction with light petroleum. Only low specific activities were obtained for the zero incubation times and these were subtracted from subsequent values.

Determination of specific activities. The light petroleum extracts were evaporated to dryness on planchets of aluminium foil which had been weighed with a microtorsion balance. The planchets were then reweighed. The samples were then counted with an end-window Geiger–Müller tube which gave 6×10^4 counts/min./μc. The self-absorption of the samples (1 mg./cm.^{2}) was considered to be negligible. The specific activity of the unsaponifiable lipid was based upon the weight of this fraction. Specific activities of saponifiable lipid and phospholipid were based upon the weights of the corresponding fatty acids obtained after hydrolysis.

Incubation of inert cells with radioactive lipoproteins. Two freshly drawn samples (50 ml. each) of whole blood were mixed with citric acid–sodium citrate–dextrose, ethylenediaminetetra-acetic acid, streptomycin and penicillin as described above. One portion (A) was stored at 2° and the other (B) was incubated for 4·5 hr. at 34·5° with sodium [*Me*-^{14}C]acetate (30 μc). The plasma was separated by centrifuging and 8 ml. of 0·16M-sodium acetate added to reduce the specific activity of unmetabolized radioactive acetate by at least 1000-fold. The cells from A were removed by centrifuging, washed with 0·16M-sodium acetate

* Part 1: James, Lovelock & Webb (1959).

and added to the plasma from B. The 'reconstituted' blood was incubated at 34° and 10 ml. samples were withdrawn at different time intervals for analysis.

RESULTS

In reporting and discussing the results, the following classifications are used. Cells include red cells, white cells and platelets. The β-lipoprotein is principally that first isolated by Oncley, Gurd & Melin (1950). The α-lipoprotein includes all plasma lipids, with the exception of β-lipoprotein, and includes chylomicra and, possibly, as yet unidentified lipoproteins. Neutral fat includes fatty acid glycerides, cholesterol esters and possibly other sterol esters. The unsaponifiable fat includes cholesterol, other sterols and possibly some esters which are resistant to hydrolysis, since prolonged saponification leads to further production of small quantities of fatty acid.

Fig. 1 shows the increase of specific activity of the neutral fat of the cells, α-lipoprotein and β-lipoprotein when whole blood is incubated with radioactive acetate. Figs. 2 and 3 show the analogous results for the phospholipids and unsaponifiable fat respectively.

In other experiments whole blood was incubated with radioactive acetate for 4–5 hr. and then the cells were replaced by non-radioactive washed cells

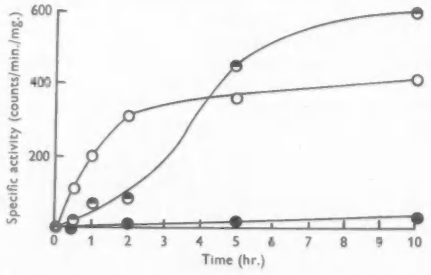

Fig. 1. Labelling of blood neutral fat on incubation with radioactive acetate. Whole blood was incubated with sodium [*Me*-^{14}C]acetate at 34·5°. ○, Neutral fat from cells; ●, neutral fat from β-lipoprotein; ◓, neutral fat from α-lipoprotein.

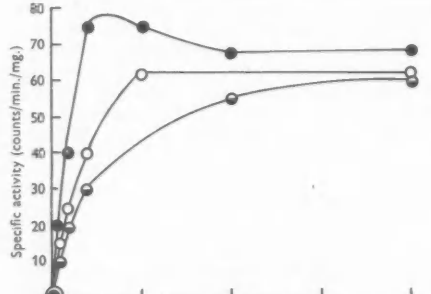

Fig. 2. Labelling of blood phospholipids on incubation with radioactive acetate. Whole blood was incubated with sodium [*Me*-^{14}C]acetate at 34·5°. ○, Phospholipids from cells; ●, phospholipids from β-lipoprotein; ◒, phospholipids from α-lipoprotein.

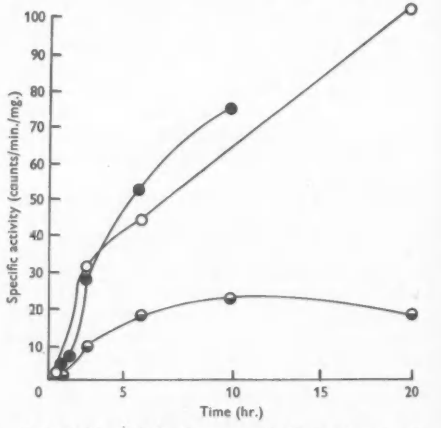

Fig. 3. Labelling of blood unsaponifiable lipid on incubation with radioactive acetate. Whole blood was incubated with sodium [*Me*-^{14}C]acetate at 34·5°. ○, Unsaponifiable lipid from cells; ●, unsaponifiable lipid from β-lipoprotein; ◒, unsaponifiable lipid from α-lipoprotein.

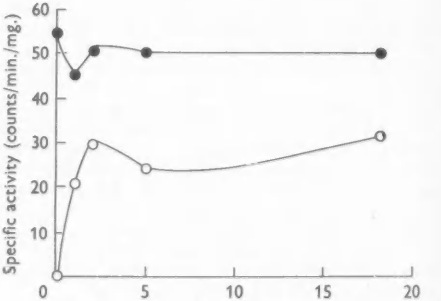

Fig. 4. Exchange of neutral fat on incubation of labelled plasma with unlabelled blood cells. Whole blood was incubated with sodium [*Me*-^{14}C]acetate at 34·5° for 4·5 hr. The cells were separated and replaced by non-radioactive cells. ○, Neutral fat from cells; ●, neutral fat from plasma.

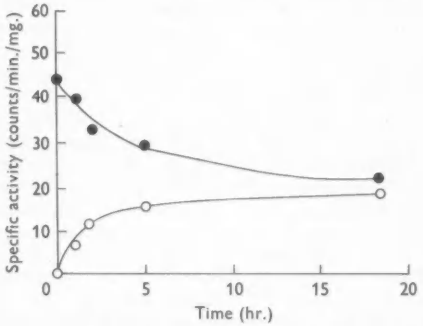

Fig. 5. Exchange of phospholipid on incubation of labelled plasma with unlabelled blood cells. Whole blood was incubated with sodium [*Me*-^{14}C]acetate at 34·5° for 4·5 hr. The cells were separated and replaced by non-radioactive cells. ○, Phospholipid from cells; ●, phospholipid from plasma.

from the same donor. The subsquent changes in specific activities of the neutral fat of the plasma and new cells are shown in Fig. 4. The corresponding changes in the phospholipids are shown in Fig. 5.

DISCUSSION

When whole blood is incubated *in vitro* with sodium [*Me*-^{14}C]acetate radioactivity is incorporated into the lipids of both the cells and the plasma lipoproteins. When plasma alone is incubated with active acetate very little radioactivity is found in the plasma lipids; this is possibly attributable to the metabolic activity of platelets remaining after the separation of the other cells. It follows that the experimental results include two general phenomena, firstly the incorporation of activity from radioactive acetate into their lipids by the red and white cells and secondly the exchange of active and inactive lipids between the cells and their plasma. In the whole animal the exchange of lipids from the plasma occurs not only with the cells but also with the other organs, particularly the liver (Hahn & Hevesy, 1939). Although this implies that the blood *in vitro* is not in a steady state so far as lipid metabolism is concerned, the isolation of the blood is nevertheless essential in distinguishing the contribution of the cells to the lipid metabolism of the whole animal.

The results of the incubation experiments indicate at first a linear rise of lipid specific activity with time for the cells, followed by a levelling off. The levelling off of specific activity could be due to the establishment of an equilibrium distribution of isotopic carbon, but it is more probably attributable to the depletion of essential metabolites in the blood or perhaps also to the accumulation of inhibiting substances. The phospholipid and the unsaponifiable lipid were observed to exchange freely with all classes of plasma lipoproteins. Neutral-fat activity was found, however, only in the α-lipoprotein-containing fraction. The free exchange of phospholipid and unsaponifiable lipid but not of neutral fat between the β-lipoprotein and the cells is consistent with a cell-like structure for β-lipoprotein, in which a central core of neutral fat is surrounded by an impermeable membrane of phospholipid, unsaponifiable lipid and protein.

Quantitative aspects. Of the added isotopic carbon 3–5 % was recovered in the lipid fractions at the time the steady level was reached. This result is particularly striking when it is recalled that 95 % of the dry weight of the cell fraction consists of haemoglobin from the red cells (Wintrobe, 1951), which is metabolically innert.

Neither the gross lipid components nor their fatty acid moieties are single entities in a chemical sense; in these circumstances the calculation of turnover and exchange rates from the experimental results is made uncertain by the dilution of radioactive substances by inert material simultaneously extracted. Nevertheless, certain approximate values for the turnover rates of the various lipid classes can be deduced.

Exchange of phospholipids. In Fig. 5 is illustrated the exchange of phospholipid when cells are suspended in plasma containing ^{14}C-labelled phospholipid. The growth and decay of phospholipid specific activity between the cells and the plasma are complementary. This experiment would suggest that the plasma phospholipid is in simple diffusion equilibrium with the same phospholipid within or at the surface of the cells. In two experiments of the type illustrated in Fig. 5, the half-life for the exchange process was 6 hr. In a separate experiment plasma phospholipids were found to be at a level of 1·3 μmoles of phosphorus/ml. of whole blood. Taking this value and 800 for the average molecular weight, the exchange of phospholipid was 0·09 mg./ml. of whole blood/hr.

The synthesis of radioactive phospholipid by the cells and its transfer to the plasma lipoproteins is illustrated in Fig. 2. The specific activity of the plasma phospholipids starts to rise immediately at the beginning of the experiment and continues to rise at a rate closely similar to those of the cellular phospholipids. The principal phospholipid of the plasma is phosphatidylcholine (77 %), whereas the cells contain only 31 % of this material (Hack, 1947). Experiments which will be reported in a later paper indicate that the phospholipid metabolism of the cells is complex, and a wide variety of phospholipid species are synthesized. In these circumstances it is hardly possible to deduce from

the experimental data any numerical values of the metabolic turnover of phospholipid by the cells. Since the increase in specific activity of the plasma lipoproteins is comparable with that of the cells, it would appear that the exchange by diffusion is rapid compared with the turnover by metabolism.

Exchange of neutral fat. Unlike the exchange of phospholipid, that of neutral fat does not appear to be determined principally by simple diffusion equilibrium. Fig. 1 shows the exchange of neutral fat between the cells and the plasma-lipoprotein fractions during an incubation experiment. The specific activity of the cellular neutral fat is seen to rise rapidly to a level approximately seven times as high as that of the phospholipid. The plasma β-lipoprotein receives little or none of this activity, even after incubation for 10 hr. After a lag period the α-lipoprotein commences to acquire radioactivity so that after 4 hr. the specific activity reaches the same level as that of the cells. The more rapid rise of activity in the cellular neutral fat than in the lipoprotein fraction would suggest that the synthesis of active neutral fat is proceeding faster in the cells than is the exchange of this lipid by diffusion. This notion is confirmed by the experiment (Fig. 4) in which cells were incubated in plasma containing ^{14}C-labelled neutral fat. No simple complementary exchange of activity occurs as with the phospholipid. Instead, the activity of the cells rises rapidly at first and then remains at a more or less steady level. Apart from a slight initial fall, little change in the plasma neutral-fat activity takes place. The values for the plasma neutral-fat specific activity here shown arise from both the active α-lipoprotein neutral fat and also the greater bulk of inert β-lipoprotein neutral-fat material. The specific activity of the exchangeable neutral fat is therefore probably three or more times that shown in Fig. 4. Failure to achieve equilibrium even after 20 hr. suggests that the exchange of neutral fat is not one of simple complementary diffusion. By making certain assumptions a value for the rate of release of neutral fat by the cells can be derived from the information shown in Fig. 1. These assumptions are first that the pool size of the α-lipoprotein neutral fat is 1 mg./ml. of plasma and second that the observed specific activity of the fat of the cells is the same as that of the fat they release. On this basis the observed rate of rise of the activity of the α-lipoprotein neutral fat requires the release of 0·16 mg. of fat/ml./hr. by the cells.

Exchange of unsaponifiable lipid. The increase of specific activity of the cellular unsaponifiable lipids and the transfer of activity to the plasma lipoproteins (Fig. 3) was similar to that observed with the phospholipids. The principal unsaponifiable lipid of all three fractions is, of course, cholesterol, and in separate experiments only a small proportion of radioactivity was found in the cholesterol when it was precipitated with digitonin. Since 90 % of the weight of the unsaponifiable lipid was cholesterol the remaining residue must have possessed a very high specific activity, and it is possible that this activity was associated with other sterols, or their intermediates.

SUMMARY

1. The incorporations *in vitro* of radioactivity from sodium [*Me*-^{14}C]acetate into the lipids of whole blood were measured.

2. Lipids from the cells, plasma β-lipoprotein and the α-lipoproteins were fractionated into unsaponifiable lipid, saponifiable lipid and phospholipid.

3. Exchanges of both 'unsaponifiable lipid' and phospholipid between cells and plasma α- and β-lipoprotein were observed.

4. There was no exchange of saponifiable lipid between the cells and β-lipoprotein. Exchange was observed between the cells and the other plasma lipids.

5. Approximate exchange rates were calculated for saponifiable lipid and phospholipid.

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The Mechanism of the Action of Androgenic Hormones on Mouse-Liver Catalase

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Adams & Burgess (1957) showed that 70–80% of the catalase activity in mouse liver was present in the large granules, the remainder being in the extraparticulate cytoplasm. The homogenization technique used before 1957 was such that the catalase measured in the suspensions was principally extraparticulate cytoplasm fraction (Adams & Burgess, 1957). Adams (1952) found that the catalase activity in such suspensions was higher when they were prepared from male mice than from female mice, and that castration of males reduced their liver catalase activity to the female level. Injection of testosterone into normal females or castrated males elevated their liver-catalase activity to about the normal male level. Adams & Burgess (1957) confirmed that the sex difference in activity was associated with the extraparticulate cytoplasm catalase fraction, and showed that the level of catalase activity in the granules was the same in both sexes. Their data suggested that different catalases were present at the two liver sites, one reacting to hormonal stimulation, the other not. Catalase activity in the erythrocytes was found to be the same in both sexes (Adams, 1956), resembling the liver-granule enzyme in this respect.

However, although many fractionation studies have been made [e.g. in rats by Greenfield & Price (1956)] no definite evidence has been found that more than one catalase is present in liver. Spectroscopic and immunological evidence obtained in a variety of species (Bonnichsen, 1947; Chance, 1950, Deutsch & Seabra, 1955) has also suggested the presence of only a single catalase in liver, which resembles closely the erythrocyte enzyme. It appears therefore that the catalase present in the granules and extraparticulate cytoplasm of liver and in erythrocytes are closely similar or identical enzymes; this conclusion provides no indication why only the extraparticulate cytoplasm fraction of liver should have a sex difference in activity.

Recently Adams & Burgess (1959*b*) showed that during the incubation of liver slices catalase migrated from the large granules into the extraparticulate cytoplasm. Catalase also migrated from the large granules into the surrounding medium when they were incubated in 0·25M-sucrose solution. No evidence could be found that this catalase migration was the result of rupture of, or damage to, the granule membranes, and it was concluded that the membranes of the large granules are permeable to catalase.

It seemed possible therefore that liver contains a single catalase, which is produced in the large granules and is then distributed between the large granules and the extraparticulate cytoplasm by migration through the granule membranes. If this hypothesis is correct, the catalase level in the extraparticulate cytoplasm will depend broadly on the granule catalase level, the permeability of the granule membranes and on the rate of catalase destruction in or loss from the extraparticulate cytoplasm. The term 'permeability' may be defined for the purpose of this paper as being that property of a membrane which governs the rate of transfer of catalase from one side to the other. At present it is not known whether the catalase transfer can occur in both directions or whether factors other than simple diffusion are involved in the process.

Such a catalase system would probably be very complex kinetically, and at present no attempt at any strict analysis can be contemplated. However, taking the simplest possible view, it may be considered that the relative levels of catalase in granules and extraparticulate cytoplasm will provide an approximate index of granule permeability.

The purpose of this paper has been to test this hypothesis by making comparative measurements of the extraparticulate cytoplasm and granule-catalase levels after the injection of various substances, and to discover whether the action of androgenic hormones is possibly to increase the permeability of the granule membranes to the enzyme.

EXPERIMENTAL

Animals. Young adult mice of the CBA strain and of the AKR Laboratory Animals Bureau substrain have been used. These two strains have similar liver-catalase activities, and the availability of sufficiently large groups has been the main factor deciding which strain has been used for which experiment. CBA mice have been bred in this Laboratory by brother–sister mating. AKR substrain mice have been

similarly bred here, or obtained from the Laboratory Animals Bureau. The diet of the animals consisted of commercial rat cubes and water (both without restriction).

Triton X 100. This non-ionic detergent was used at a final concentration of 0·25 % to disrupt large granules and liberate their catalase activity into solution.

Homogenization of liver. The preparation of a 12-stroke homogenate has been previously described (Adams & Burgess, 1957). In the present work ethanol (0·01 M) has been present in all catalase-containing solutions and suspensions to prevent losses in activity through the formation of the catalase–hydrogen peroxide complex II (Chance, 1950; Adams & Burgess, 1959*a*). Catalase measurements were made of the following preparations: (1) 12-stroke homogenate + Triton (giving total catalase); (2) extraparticulate cytoplasm (EPC) fraction (supernatant after centrifuging a 12-stroke homogenate at 10 000 *g* for 20 min.); (3) granule fraction [sediment from (2) after resuspending it in Triton solution to the original volume].

Estimation of liver-catalase activity. Catalase was estimated as previously described (Adams, 1950, 1952). The catalase activities of the various fractions have been referred to the nitrogen content of the whole homogenate from which they were prepared. One advantage of this is that the ratio of the catalase activities in granules and EPC is independent of the concentration of any other tissue component.

Chemical substances. (Cysteine hydrochloride, thiourea and methylbis-(β-chloroethyl)amine (NM) were separately dissolved in sodium chloride solution (0·9 %) and brought to pH 7·0 by the addition of sodium bicarbonate solution, the pH being measured with a Pye pH meter and glass electrode standardized according to the maker's instructions. They were then injected intraperitoneally.

Individual doses of testosterone, 17-methyltestosterone and 3′-methyl-4-dimethylaminoazobenzene (MDAB) were weighed on a torsion balance, each dose then being placed separately on a siliconized white tile. Each dose was moistened with 0·9 % sodium chloride and then implanted intraperitoneally with a trocar and cannula.

RESULTS

Normal animal. Table 1 gives the total, granule and EPC liver-catalase activities found in male and female AKR and CBA mice. The results show that the granule-catalase activity was very similar in males and females in each strain, whereas the EPC catalase level was much higher in the males than in the females. The sex difference in EPC catalase activity was much higher in untreated AKR and CBA mice than in the stock albino strain used for the previous hormone experiments (Adams, 1952, 1956). Thus the granule/EPC catalase distribution ratio was about 4·9 in the females and 3·4 in the males.

Effect of the injection of non-androgenic substances. Fig. 1 shows the effect of the injection of single doses of cysteine on the total, granule and EPC catalase levels, and on the granule/EPC catalase distribution ratio, of CBA female mice. The results show that depressions of total catalase activity occurred within 24 hr., followed by a return to normal. The extent of the depression and the time needed for recovery increased with increasing dose of cysteine. The shaded areas on the graphs are in fact approximately proportional to dose. Little or no change was seen in the granule/EPC distribution ratio.

Cysteine is not a direct inhibitor of catalase: however, when solutions of catalase and cysteine were mixed, losses in activity occurred due to the combination of the enzyme with small quantities of hydrogen peroxide produced by auto-oxidation (complex II, Chance, 1950). To examine the possibility that the catalase loss in the livers of cysteine-treated animals was due to complex II formation *in vivo*, samples of the homogenates prepared from the 48 hr. group given the largest dose of cysteine were incubated at 38° for 2 hr. Adams & Burgess (1959*b*) found that complete regeneration of catalase activity from solutions containing complex II was achieved under these conditions in the presence of ethanol. However, no rise in activity was observed in the liver homogenates made from the cysteine-treated animals (Table 2).

The effect of the implantation of two doses of MDAB into AKR females, and the larger dose into AKR males, is shown in Fig. 2. Once again catalase activity was rapidly depressed, the amount of the depression (extent × duration) being approximately proportional to dose in the two cases. The granule/EPC distribution ratio was only slightly affected in either sex except on the third day after the injection of the smaller dose into females, when the

Table 1. *Liver-catalase activities and intracellular distribution ratio (granule/extra particulate cytoplasm) in normal male and female CBA and AKR mice*

Catalase activities are expressed in arbitrary units/mg. of N as arithmetic means ± S.E.M. Eight animals were used in each group.

CBA mice	Total activity	Granule activity	EPC activity	Granule/EPC ratio
♂	625 ± 10·5	480 ± 7·9	140 ± 4·1	3·4
♀	575 ± 10·2	475 ± 8·4	98 ± 2·8	4·8
AKR mice				
♂	640 ± 12·0	490 ± 11·2	140 ± 3·9	3·5
♀	600 ± 10·2	500 ± 9·0	100 ± 3·1	5·0

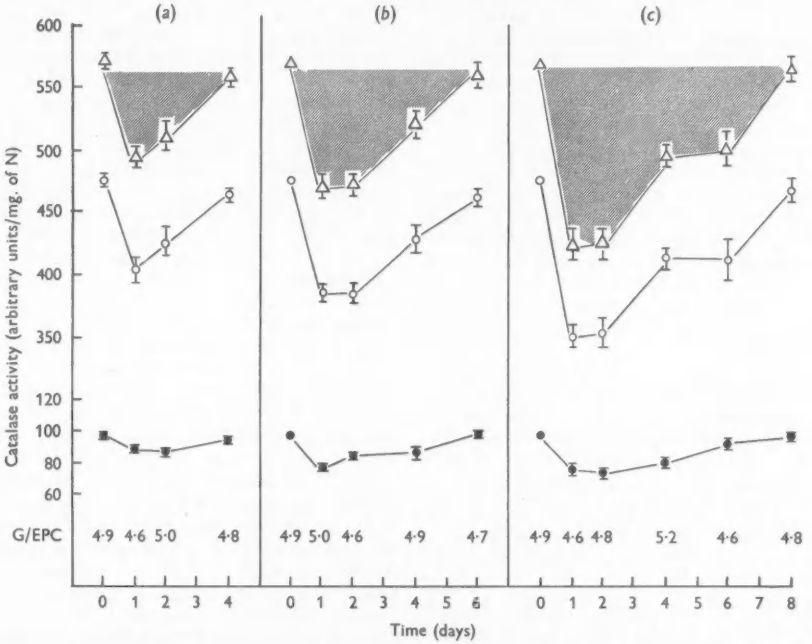

Fig. 1. Action of single doses of cysteine on the liver-catalase activity and intracellular-catalase distribution (granule/extraparticulate cytoplasm; G/EPC) of CBA female mice. △, Total catalase; ○, granule catalase (G); ●, extraparticulate-cytoplasmic catalase (EPC). (*a*) 20 μmoles. (*b*) 50 μmoles. (*c*) 125 μmoles. Controls at 0 days. The same control group was used for all three graphs. Twelve control and eight treated mice were used in each group. Results in this and subsequent figures are given as arithmetic means ± S.E.M.

Table 2. *Total catalase activity in the livers of mice before and after incubation of homogenates with* 0·01 M-*ethanol for* 2 *hr. at* 38°

(*a*) CBA female mice 48 hr. after the injection of 125 μM-cysteine; (*b*) AKR female mice 48 hr. after the injection of 1 μmole of NM. Results are given in arbitrary units/mg. of N as arithmetic means ± S.E.M. Eight animals were used in each group, with twelve controls.

	Control catalase activity	Catalase activity of liver of treated animals: Before incubation with ethanol	Catalase activity of liver of treated animals: After incubation with ethanol
(*a*)	540 ± 11·2	425 ± 12·5	430 ± 11·0
(*b*)	590 ± 10·5	210 ± 11·5	220 ± 12·1

ratio rose to 5·7. However, this result may be slightly aberrant since the larger dose gave a distribution ratio of only 5·4 after 3 days.

Thiourea (10 μmoles) injected into male and female CBA mice affected their catalase activity in a similar way to MDAB. The results were almost identical with those obtained with the higher dose of MDAB.

Fig. 3 shows the results of the injection of various single doses of NM into female AKR mice and of one single dose into males. This substance was extremely toxic; the mice receiving the largest dose (1 μmole) did not survive beyond 48 hr., and at this time had lost about 70 % of their liver-catalase activity. The intermediate dose (0·2 μmole) resulted in the death of some of the treated animals. As with the substances already described the depressions in catalase activity were related to dose. However, the granule/EPC catalase distribution ratio was altered from 5·0 in the females and 3·3 in the males to a new value of 4·2–4·4 in both sexes.

Erythrocyte-catalase measurements were made on the group of female mice 48 hr. after the injection of the largest dose of NM, when their liver-catalase activity was about 30 % of normal (Fig. 3), and their erythrocyte-catalase activity was

found to be unchanged. Samples of liver homogenate from these mice were also incubated with ethanol (cf. experiments with cysteine, described above). As shown in Table 2 no regeneration of catalase activity was observed.

Incubation of homogenates with methylbis-(β-chloroethyl)amine in vitro. Liver homogenates were prepared with 300 strokes of a Ten Broeck grinder, which liberates most of their catalase activity (Adams & Burgess, 1957). They were centrifuged at 10 000 *g* for 20 min. and the supernatants were then incubated with various concentrations (up to 0·01 M) of NM at 0° for 2 hr. The NM was dissolved in water and brought to pH 7·0 with sodium bicarbonate before use. As shown in Table 3, little inhibition was seen.

Effect of the injection of androgenic hormones. In preliminary experiments it was found that testosterone administered to female mice of the two strains used caused a rise in EPC-catalase level, but to a point below the normal male level. Further experiments showed that 17-methyltestosterone would elevate the EPC catalase level in females to, or to a little above, the male level.

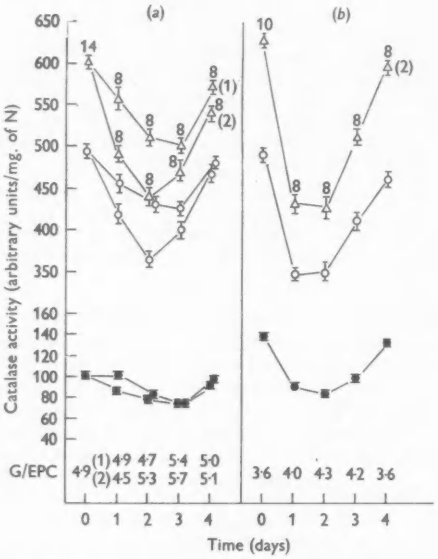

Fig. 2. Action of single doses of 3′-methyl-4-dimethylaminoazobenzene on the liver-catalase activity and intracellular-catalase distribution of AKR mice. △, Total catalase; ○, granule catalase; ●, EPC catalase. (*a*) Females; (*b*) males. (1) 10 μmoles. (2) 20 μmoles. Numbers of animals in the groups are given by the figures at the head of the S.E. limits.

The action of single injections of 17-methyltestosterone on the liver-catalase activity of female CBA mice is shown in Fig. 4. So far as total catalase activity is concerned, the results closely resembled those obtained with cysteine (Fig. 1), i.e. transient catalase depressions proportional to dose. However, unlike cysteine, striking effects were seen on the granule/EPC-catalase distribution ratio. This changed from the female value to the male value 48 hr. after the injection, and independently of dose. With the largest two doses the new ratio was maintained for several days, during which time the granule catalase returned to normal and the EPC activity rose to the male level.

Testosterone produced results on injection (Fig. 5) similar to those obtained with 17-methyltestosterone. As previously mentioned, however, the EPC-catalase activity, although elevated, did not reach the male level, the granule/EPC-catalase distribution ratio falling to a value intermediate between that of males and that of females.

Fig. 6 shows the effect of the injection of 17-methyltestosterone into males. Over the first few days the results closely resembled those obtained after the injection of cysteine, thiourea and MDAB, i.e. a transient fall in catalase activity in both fractions, with little or no alteration in the granule/EPC distribution ratio. From the sixth to the eighth days the EPC-catalase level fell, but not the granule level, the distribution ratio approaching the female value. By the eleventh day the EPC level returned to normal.

DISCUSSION

All the substances tested caused losses in total catalase activity after injection, the activity usually returning to normal after 4–7 days. The losses were approximately proportional to the dose of injected material. The results suggested that the erythrocyte catalase remained unchanged under the applied conditions and that the losses in liver catalase were not due either to direct inhibition or to the formation of complex II (Chance, 1950).

It seems reasonable to suppose that the losses in total catalase activity are due to hydrogen peroxide produced during the metabolism of the injected material, and are consequently of a non-specific nature.

The catalase activity after the injection of the highest doses of the steroids has shown a tendency to rebound to a higher than normal level. This rebound, coupled with the other results presented, suggests that the turnover rate of catalase is high, and that the synthetic mechanisms are capable of responding rapidly to a sudden demand. Also, Adams & Burgess (1959*b*) found that 70 % of their catalase could migrate from isolated granules to the

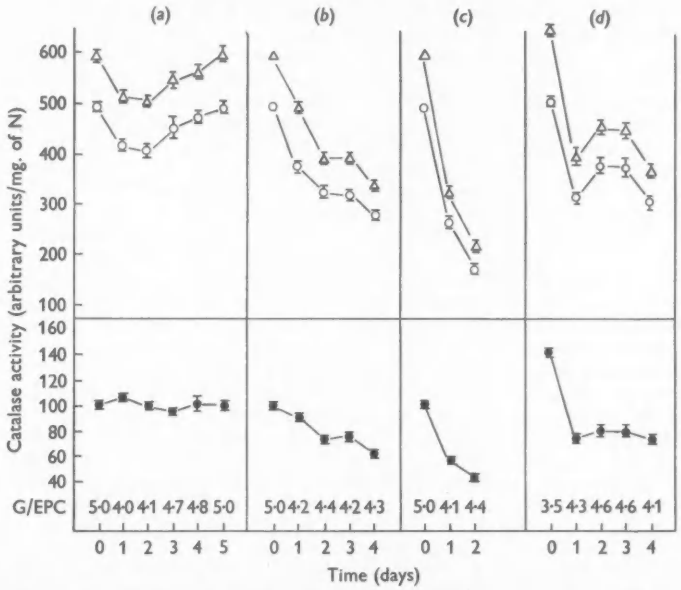

Fig. 3. Action of single doses of methylbis-(β-chloroethyl)amine on the liver-catalase activities and intracellular-catalase distribution of AKR mice. △, Total catalase; ○, granule catalase; ●, EPC catalase. (*a*), (*b*) and (*c*), female mice receiving 0·05, 0·2 and 1·0 μmole respectively; (*d*) male mice receiving 0·2 μmole. Twelve control and eight treated animals were used in each group. The same control group was used for graphs (*a*), (*b*) and (*c*).

Table 3. *Action of methylbis-(β-chloroethyl)amine on liver catalase* in vitro *during incubation for up to 1 hr. at 0°*

A 300-stroke homogenate of liver in water (Adams & Burgess, 1957) was centrifuged at 10 000 *g* for 20 min., and the supernatant diluted tenfold with water. Samples were mixed with aqueous solutions of NM, brought to pH 7·0 with bicarbonate, to give the final concentrations stated.

Time of incubation (min.)	Concn. of NM (mM) 0 (Control)	1	5	10
	Catalase activity (arbitrary units/mg. of N)			
10	519	525	570	548
	546	558	540	540
60	540	575	545	520
	556	560	550	510

surrounding medium within 30 min. The system would therefore seem well fitted to deal with the sudden intracellular production of hydrogen peroxide.

The hypothesis given in the introduction to this paper was, briefly, that catalase is produced within the large granules and then migrates into the EPC. The results obtained have shown clearly that the granule catalase may be destroyed and regenerated very rapidly, and since most of the enzyme is found in the granules they must be at least the major site of catalase production. Catalase migration cannot easily be demonstrated *in vivo*, although its occurrence *in vitro* has been shown (Adams & Burgess, 1959*b*). However, the effects of the non-androgenic substances suggest very strongly that the catalase levels in granules and EPC are interdependent. Thus changes in granule-catalase level have always been associated with similar changes in EPC-catalase level, over the whole range of doses and time. After the injection of cysteine, thiourea and MDAB, the granule/EPC-catalase distribution ratio remained at the control value in both males and females. There seem only two possible explanations of this result. Either there is a single catalase which migrates in the way already proposed, or there are two separate catalase systems, one in the granules and one in the EPC, which are affected to the same proportional extent, independently of substance, dose and time. The pattern obtained after the injection of NM provided evidence in favour of the first alternative. In the females, the granule/EPC distribution ratio was lowered, from the control value of 5·0 to a

new value of about 4·2 which was maintained independently of dose and time, and, in the males, the distribution ratio was raised from the control value of 3·3 to about 4·4, i.e. to about the same value as in the females. These results are not easily explained in terms of separate granule and EPC-catalase systems. They may, however, be readily explained by supposing that part of the action of NM is to alter the rate of catalase migration from granules to EPC, whereas cysteine, MDAB and thiourea do not alter the rate of migration.

An explanation of the effects of androgens on catalase may be given in similar terms. The granule/EPC distribution given in Table 1 would be expected if (1) the EPC-catalase level depended on the granule-catalase level and (2) the rate of catalase migration was higher in the males. The action of testosterone and 17-methyltestosterone on the total catalase activity of females closely resembled that of cysteine, for example. However, within 48 hr. after the injection of 17-methyltestosterone in any of the doses used, the granule/EPC-catalase distribution ratio changed to the male value, and remained there during the subsequent rise in EPC level.

Testosterone gave similar results. However, the granule/EPC distribution ratio changed to a value intermediate between the normal male and female values, reflecting the fact that the EPC-catalase activity, although elevated, did not reach the male level.

The injection of 17-methyltestosterone into males resulted, over the first 4 days, in a fall followed by a rise in catalase activity in both fractions, without change in the distribution ratio. This result closely resembles those obtained with cysteine, thiourea and MDAB in both males and females. From the sixth to the eighth day the granule/EPC distribution ratio approached the female value. It seems probable that this was due to a temporary suppression of the endogenous androgen secretion by the large administered dose.

The data presented, when taken in conjunction with the evidence that the large-granule membranes are permeable to catalase (Adams & Burgess, 1959*b*), are consistent with the following: (1) Catalase, largely produced in the granules, is

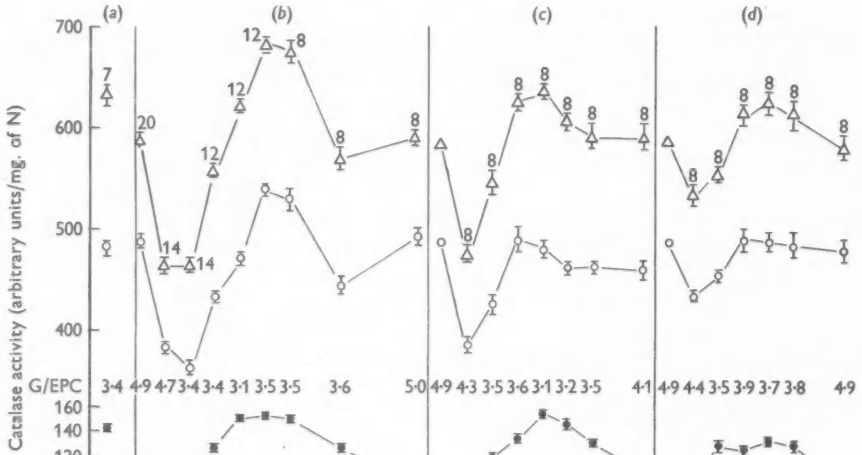

Fig. 4. Action of single doses of 17-methyltestosterone on the liver-catalase activity and intracellular-catalase distribution of CBA female mice. △, Total catalase; ○, granule catalase; ●, EPC catalase. (*a*) A comparable group of normal male mice; (*b*), (*c*) and (*d*), female mice receiving 8·0, 3·0 and 0·8 μmoles respectively. The same control group was used for (*b*), (*c*) and (*d*). Numbers of animals in the groups are given by the figures at the head of the S.E. limits.

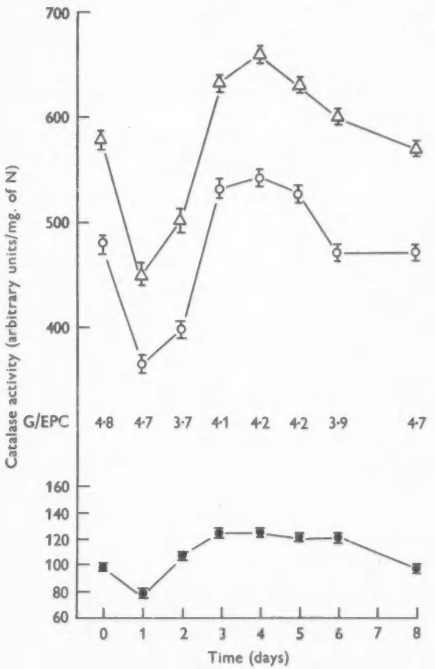

Fig. 5. Action of a single dose of 8 μmoles of testosterone on the liver-catalase activity and intracellular-catalase distribution of CBA female mice. △, Total catalase; ○, granule catalase; ●, EPC catalase. Twelve control and eight treated animals were used in each group.

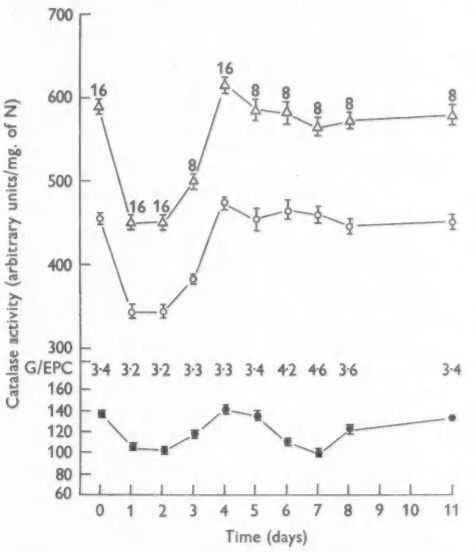

Fig. 6. Action of a single dose of 8 μmoles of 17-methyltestosterone on the liver-catalase activity and intracellular-catalase distribution of male CBA mice. △, Total catalase; ○, granule catalase; ●, EPC catalase. The numbers of animals in the groups are given by the figures at the head of the S.E. limits.

distributed between the granules and EPC by migration through the granule membranes. (2) Androgenic hormones influence the intracellular distribution of catalase by increasing the rate of migration. (3) It is reasonable to suppose that the increased rate of migration is the result of an increased permeability of the granule membranes, brought about by the hormones. (4) Methyltestosterone increases the granule permeability to a greater extent than testosterone.

This theory also provides a plausible explanation of phenomena described in previous publications. Firstly, the absence of a sex difference in erythrocyte-catalase level (Adams, 1956). Hormones could not influence catalase distribution in the erythrocyte in the way suggested for liver. Secondly, copper and manganese deficiency in females resulted in an elevation of EPC-catalase activity resembling that observed after the injection of testosterone (Adams, 1953). This may be explained by supposing that trace metals are also implicated in the control of the permeability of granule membranes.

SUMMARY

1. A study has been made of the effect of the injection of a number of substances, including androgenic hormones, on the total catalase activity and the intracellular-catalase distribution between large granules and extraparticulate cytoplasm in the livers of mice.

2. All the substances tested produced depressions of catalase activity, approximately proportional to dosage, usually followed by a return to normal after a few days.

3. These catalase losses are probably of a non-specific nature, and it is suggested that they may be due to hydrogen peroxide produced during the metabolism of the injected material.

4. Cysteine, thiourea and 3-methyldimethylaminoazobenzene caused losses in catalase activity without markedly affecting the intracellular-catalase distribution.

5. Males and females have a different intracellular-catalase distribution. In males the granule/extraparticulate-cytoplasmic distribution ratio is about 3·3, in females it is about 4·9.

6. A nitrogen mustard altered the catalase distribution ratio in males and females to the same value, intermediate between their normal values.

7. 17-Methyltestosterone injected into females caused a change in the catalase distribution ratio to the male value. Testosterone behaved similarly, but the distribution ratio was altered to a point intermediate between the male and female values.

8. 17-Methyltestosterone injected into males did not affect the distribution ratio.

9. It is concluded that the catalase levels in the granules and extraparticulate cytoplasm are interdependent; that catalase probably migrates from the granules into the extraparticulate cytoplasm; and that androgenic hormones influence the rate of migration in a way which suggests that they increase the permeability of the granule membranes to catalase.

The expenses of this research were partly defrayed out of a block grant from the British Empire Cancer Campaign. My thanks are due to Dr M. H. Salaman for his interest and to Miss I. Harris for skilled technical assistance. I also wish to thank Dr E. Margoliash very cordially for his suggestion to me that thiourea would be an interesting substance to study as a catalase inhibitor *in vivo*. Thanks are due to Charles Lennig and Co. for the gift of Triton, and to Boots Pure Drug Co. Ltd. for the gift of methylbis-(β-chloroethyl)amine.

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The Behaviour of Liver Mitochondria Isolated from Rats with Different Body Temperatures after Limb Ischaemia or after Injection of 3:5-Dinitro-*o*-Cresol

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The liver is one of the main sites of heat production in the body (Federov & Shur, 1942; Birnie & Grayson, 1952) and the heat formed there mainly arises during the oxidations carried out by the mitochondria. It is therefore possible that changes in hepatic heat production might reflect alterations within the mitochondria or be due to variations in the amount of substrate being fed to mitochondria whose potentialities had remained unaltered.

The changes in liver temperature on simple exposure of the rat to different environmental temperatures are quite small (Stoner, 1958*a*), although the changes in overall metabolic rate may be large and rapid (Depocas, Hart & Héroux, 1957). Exposure to cold increases heat production but, according to Panagos, Beyer & Masoro (1958), mitochondrial changes occur only as the rat becomes acclimatized.

Much greater changes in liver temperature occur in rats which have been injured by limb ischaemia, particularly if the environmental temperature is also altered (Stoner, 1958*a*). The possibility of mitochondrial changes in these rats is of special interest as several workers (Strawitz & Hift, 1956*a*, *b*; Packer, Michaelis & Martin, 1958; Hift & Strawitz, 1958) have sought to relate the metabolic decline and death of the injured rat to alterations in the mitochondria.

Fatal doses of 3:5-dinitro-*o*-cresol, which has the same biochemical action as 2:4-dinitrophenol, produce very high liver temperatures in the rat (Stoner, 1956) and the acute effects of this compound have also been investigated.

Experience in the production of a highly reproducible preparation of liver mitochondria (Aldridge, 1957) has allowed us to seek a possible association between acute changes in temperature (heat production) and the biochemical behaviour of the isolated mitochondria. It was found that except in rats treated with 3:5-dinitro-*o*-cresol large changes in liver temperature were unaccompanied by similar changes in the behaviour of the isolated mitochondria.

METHODS

Animal technique. Male albino rats (200–250 g. each) of the Porton strain fed on M.R.C. diet 41 b (Bruce & Parkes, 1956) were used, food being provided without restriction until the start of the experiment. Bilateral hind-limb tourniquets (Rosenthal, 1943) were applied under ether and the controls were given a similar short period of ether anaesthesia. The tourniquets were kept on for 4 hr. The changes in environmental temperature were made as before (Stoner, 1958*a*). Two rats, an experimental and a control, were used in each test so that both were subjected to the same environmental conditions. To avoid direct trauma to the liver the liver temperature itself was not measured but was inferred from the 'high' colon temperature determined with a mercury thermometer inserted through the anus for a distance of 8 cm. This temperature is only slightly below that in the liver (Stoner, 1958*a*).

In two experiments 3:5-dinitro-*o*-cresol was injected subcutaneously as a 1% (w/v) solution in 0·1 M-Na_2CO_3.

Preparation of mitochondria. The rats were killed by decapitation and the liver mitochondria were isolated as previously described (Aldridge, 1958). The mitochondria from the control and experimental rats were prepared at the same time, the times of deaths of the two animals being separated by about 10 min.

Manometric technique. The reasons for the composition and the pH of the medium have been previously discussed (Aldridge, 1957). Each flask contained 3 ml. of a solution containing adenosine 5-phosphate (1·15 mM), adenosine triphosphate (ATP; 1·06 mM), KCl (0·1 M), $MgCl_2$ (14 mM), ethylenediaminetetra-acetic acid (1·0 mM), potassium phosphate (15 mM), sucrose (0·03 M), and substrates which were all 0·01 M except fumarate (1·0 mM). For studies of oxidative phosphorylation each flask contained, in addition, glucose (180 μmoles), glycylglycine (50 μmoles) and hexokinase (200–400 units). This medium was adjusted to pH 6·7–6·8 with KOH, a glass electrode being used, and all experiments were carried out at 37°. Carbon dioxide was absorbed by 0·15 ml. of 5 N-KOH; the gas phase was air. The mitochondria were suspended in 0·3 M-sucrose. In all experiments, 0·30 ml. of mitochondrial suspension, equivalent to 0·15 g. wet wt. of original liver, was used.

Determination of adenosine triphosphatase activity. Each beaker contained 3 ml. of a solution containing ATP (3 mM), KCl (0·1 M), $MgCl_2$ (14 mM), ethylenediaminetetra-acetic acid (1·0 mM), glycylglycine (16·7 mM) and sucrose (0·03 M). This solution was adjusted to pH 6·7–6·8 with KOH. The beakers were shaken at 37°. After temperature equilibration mitochondria were added and the mixture was incubated for 10 min. The reaction was stopped by the addition of 0·5 ml. of 65% (w/w) $HClO_4$ and inorganic phosphate was determined.

Analytical methods. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925). Mitochondrial protein was measured by the biuret method of Robinson & Hogden (1940), modified as described by Aldridge (1957), and has been expressed as mg. of albumin. 3:5-Dinitro-*o*-cresol was determined by the method of Parker (1949).

Special chemicals and reagents. The following chemicals were obtained from the sources indicated: glycylglycine, sodium pyruvate (Roche Products Ltd.); adenosine 5-phosphate, disodium salt of ATP (Sigma Chemical Co., St Louis, Mo., U.S.A.); sodium fumarate, 3:5-dinitro-*o*-cresol and 2:4-dinitrophenol (British Drug Houses Ltd.). All free acids were neutralized to approx. pH 6·8 with KOH before being used. Potato apyrase was prepared by the method of Lee & Eiler (1951). This preparation liberated 1100 μg.atoms of inorganic phosphate/hr./ml. from the medium used for adenosine triphosphatase assay at 37°. Hexokinase was prepared from baker's yeast by a modification by V. H. Parker (unpublished work) of the method of Berger, Slein, Colowick & Cori (1946). The preparation was taken to the equivalent of their step 3 a and when assayed by their procedure (but at 37° instead of 30°) had an activity of 3500 units/ml.

Units used. The oxidative activity of the mitochondria (q_{O_2}) is expressed as μl. of O_2/mg. of protein/hr. Adenosine triphosphatase activity is given as μg.atoms of P liberated/mg. of protein/hr.

RESULTS

Controls

Since each control rat was exposed to the same environmental conditions as the experimental one the controls may be divided into three groups (Tables 1 and 2). First there is the group from Expts. 1, 4, 12 and 13, in which the rats were kept at an air temperature of 16–21°. The controls in Expts. 2 and 3 were exposed to an air temperature of 3°. This has previously been found to raise the liver temperature about 0·6° without affecting its blood flow (Stoner, 1958*a*). According to the work of Depocas *et al.* (1957) the overall metabolic rate of these rats would have been increased 1·7-fold. In the third group in Expts. 5–10 the environmental temperature was raised to the so-called critical level for the rat. Under these conditions the liver temperature falls slowly and there is a 31% decrease in the rat's total oxygen consumption compared with that at 20° (Stoner, 1958*a*). In one rat of this group (Expt. 11) the air temperature was raised to 36°, which is about the limit of tolerance, and at this air temperature the liver temperature rises.

Despite the different metabolic rates of these control rats no differences were found in the behaviour of the mitochondria isolated from them (Tables 1 and 2). In respect of their oxygen uptake and its stimulation by apyrase, their adenosine triphosphatase activity and its stimulation by 2:4-dinitrophenol, and their protein content, there was no consistent difference between liver mitochondria from rats at high and low environmental temperatures and those from rats at air temperatures of 16–21°. The results in the last group were within the range consistently found for such rats in this Laboratory over the past 2½ years.

Effect of limb ischaemia

More striking changes in liver temperature were produced by subjecting rats to limb ischaemia (Tables 1 and 2).

A 4 hr. period of bilateral ischaemia of the hind limbs at an air temperature of 18–22° gives an 85 % mortality rate with a survival time of about 13 hr. (Stoner & Threlfall, 1959). After removal of the tourniquets the liver temperature falls steadily in both fatal cases and survivors. After about 4 hr. its recovery begins in those which survive but the fall continues in those which die (Stoner, 1958*a*). In the rats which die, the liver blood flow does not fall significantly until about 4 hr. after removal of the tourniquets, i.e. it would be greatly below the normal level in Expt. 1, where the rat was moribund, but not in Expt. 4. Exposing the rats to an environmental temperature of 3° some time after removal of the tourniquets, as in Expts. 2 and 3, increases the mortality rate to 100 % and greatly accelerates the fall in liver temperature without markedly affecting its blood flow (Stoner, 1958*a*). In Expts. 2 and 3 the rats were at an air temperature of 3° for 55 and 90 min. respectively before they were killed.

Despite these gross falls in colon (liver) temperature there were no correspondingly large changes in the behaviour of the isolated mitochondria (Table 1 and 2).

After limb ischaemia the liver temperature can be made to rise if the rat is kept in an environmental temperature of 30° during and after a 4 hr. period of hind-limb ischaemia (Stoner, 1958*a*). When the tourniquets are removed the liver temperature rises until just before death, when it falls suddenly. The liver blood flow falls rapidly when the tourniquets are removed. This procedure increases the mortality rate to 100 % and shortens the survival time to about 2 hr. (Stoner & Threlfall, 1959). This was done in Expts. 5, 6, 8 and 10. In Expt. 7 the environmental temperature was not raised until after the period of limb ischaemia. The

Table 1. *Oxygen uptake of mitochondria, from control and experimental rats, with pyruvate as substrate*

Measurements were made as described under Methods. 'With apyrase' indicates addition of apyrase of activity corresponding to liberation of 110 μg.atoms of inorganic P/hr. under assay conditions described in the Methods section. C, Control rats; E, experimental rats. In Expts. 1–11 the experimental rats were subjected to a 4 hr. period of bilateral hind-limb ischaemia. The tourniquets were applied under ether anaesthesia and the controls were given a similar short anaesthesia. Dose of 3:5-dinitro-*o*-cresol: 25 mg./kg. body wt. subcutaneously.

Expt. no.	Environmental temp.	Interval after removal of tourniquets	Final colon temp.	Oxygen uptake (q_{O_2}) No apyrase (*a*)	Oxygen uptake (q_{O_2}) With apyrase (*b*)	Stimulation (ratio *b*/*a*)
			Ischaemic shock			
1C	16°	—	38·0°	24·6	90	3·7
1E	16	18 hr.	22·0	29·5	109	3·7
2C	18/3*	—	38·6	29·0	111	3·8
2E	18/3	3 hr. 55 min.	25·3	35·0	121	3·5
3C	17/3	—	38·0	26·9	97	3·6
3E	17/3	4 hr. 15 min.	26·0	27·2	104	3·8
4C	20	—	37·2	25·1	107	4·3
4E	20	2 hr. 38 min.	32·7	27·8†	106†	3·8
5C	30	—	37·8	30·0	106	3·5
5E	30	1 hr.	38·3	34·8	112	3·2
6C	30	—	—	26·0	—	—
6E	30	38 min.	39·2	28·0	—	—
7C	20/30	—	37·8	28·2	87	3·1
7E	20/30	1 hr. 16 min.	39·2	31·0	117	3·8
8C	30	—	—	—	85	—
8E	30	50 min.	—	—	87	—
9C	30	—	—	30·0	89	3·0
9E	30	45 min.	—	20·0	87	4·3
10C	30	—	—	23·0	87	3·8
10E	30	30 min.	—	25·0	86	3·4
11C	36	—	39·0	23·8	88	3·7
11E	36	15 min.	40·5	29·8	102	3·4
			3:5-Dinitro-*o*-cresol			
12C	18	—	38·0	18·5	89	4·8
12E	18	—	42·0	49·0	111	2·3

* 18/3 indicates a period at 18° followed by one at 3° (see text).
† Calculated from mean recovery of mitochondrial protein (cf. Table 3).

rise in colon temperature between removal of the tourniquets and killing the rats was 0·7°, 2·2° and 2·2° in Expts. 5–7 respectively. It was not measured in Expts. 8–10 but the average rise in liver temperature during this period in previous experiments was 0·6° (Stoner, 1958*a*).

Table 2. *Adenosine triphosphatase activity and protein of mitochondria from control and experimental rats*

Measurements were made as under Methods. C, Control rats; E, experimental rats. For treatment of rats see Table 1 and text. DNP, 2:4-Dinitrophenol (30 μM).

Expt. no.	Final colon temp.	Protein (mg./g. wet wt. of liver)	Adenosine triphosphatase: No DNP (μg.atoms of P/mg. of protein/hr.)	Adenosine triphosphatase: With DNP (μg.atoms of P/mg. of protein/hr.)
		Ischaemic shock		
1C	38·0°	30·8	0·49	11·7
1E	22·0	29·3	0·16	12·3
2C	38·6	28·5	0·26	—
2E	25·3	30·9	0·07	—
3C	38·0	27·8	0·14	13·0
3E	26·0	30·1	0·08	13·2
4C	37·2	28·2	0·16	—
4E	32·7	—	0·04*	—
5C	37·8	24·7	0·44	12·8
5E	38·3	23·9	0·35	13·0
6C	—	25·5	0·45	14·4
6E	39·2	28·9	0·11	13·8
7C	37·8	27·8	0·99	—
7E	39·2	30·5	0·35	—
8C	—	30·1	—	—
8E	—	39·0	—	—
9C	—	28·9	—	—
9E	—	34·0	—	—
10C	—	34·0	—	—
10E	—	38·2	—	—
11C	39·0	27·4	0·41	13·5
11E	40·5	24·7	0	14·8
		3:5-Dinitro-*o*-cresol		
12C	38·0	30·5	0·17	13·1
12E	42·0	30·1	5·20	11·8
13C	38·3	29·5	0·14	12·6
13E	41·5	25·4	3·04	12·1

* Calculated from mean recovery of mitochondrial protein (cf. Table 3).

Again these temperature changes were not reflected in the behaviour of the liver mitochondria *in vitro* (Tables 1 and 2). Their oxygen uptake with and without added apyrase, their adenosine triphosphatase activity with and without the addition of 2:4-dinitrophenol and their protein content was not strikingly different from those from the controls or from the injured rats with low liver temperatures. In Expts. 9 and 10 it was also found that the oxygen uptake with L-glutamate and succinate as substrates, both with and without apyrase, was likewise unchanged after limb ischaemia.

A higher liver temperature was produced by keeping the rat in an air temperature of 36° during and after the period of limb ischaemia (Expt. 11). This reduces the survival time after removal of the tourniquets to about 30 min. (Stoner & Threlfall, 1959). Under these conditions the liver temperature rises during the period of limb ischaemia with a further rise afterwards. Liver blood flow is depressed while the tourniquets are in place and falls precipitously when they are removed (Stoner, 1958*a*). Although in Expt. 11 conditions in the liver must have been grossly abnormal for about 3 hr. before it was removed, the subsequent behaviour of the mitochondria *in vitro* was not affected.

Inspection of the results in Tables 1 and 2 shows that despite the different environmental conditions there is a close similarity among those obtained in the controls and among those in the injured rats. In view of this the results in the two groups have been pooled, when certain overall differences become apparent (Table 3). The oxygen uptake by the mitochondria from the injured rats, both with and without apyrase, is significantly higher than in the control mitochondria but the degree of stimulation by apyrase is the same in the two groups. Similarly, the adenosine triphosphatase activity of the 'experimental' mitochondria is less than that of the 'controls', but in both cases the same degree of activity can be produced by the addition of an amount of 2:4-dinitrophenol which is known

Table 3. *Pooled results on mitochondria from control rats and rats in ischaemic shock (Expts. 1–11)*

Values given are means ±S.D., with number of determinations in parentheses. Significance of differences were tested by Student's *t* test.

Assay	Control	Experimental	*P*
Protein (mg./g. wet wt. of liver)	28·6±2·5 (12)	30·9±4·7 (11)	0·08
Oxygen uptake, no apyrase (q_{O_2})	24·1±3·5 (11)	29·1±2·9 (10)	0·001
Oxygen uptake, with apyrase ($\bar{q}_{O_2}$)	94·2±9·4 (11)	103·8±12·2 (11)	0·03
Stimulation (ratio)	3·73±0·52 (11)	3·66±0·32 (10)	0·33
Adenosine triphosphatase, no DNP (μg.atoms of P/mg. of protein/hr.)	0·39±0·26 (9)	0·145±0·13 (8)	0·02
Adenosine triphosphatase, with DNP (μg.atoms of P/mg. of protein/hr.)	13·08±0·88 (6)	13·15±1·07 (6)	0·46

(Parker, 1958) to produce maximal stimulation. Varying the order in which the rats were killed eliminated the possibility that these differences were related to the intervals between death and the commencement of the assays *in vitro*.

Effect of 3:5-*dinitro*-o-*cresol*

Very high liver temperatures can be produced by fatal doses of 3:5-dinitro-*o*-cresol. The rise occurs during the period of hyperventilation and the liver blood flow shows variable changes, usually falling (Stoner, 1956). In Expts. 12 and 13 the rats were killed and the liver mitochondria isolated when the effects were judged to be maximal, 50 and 73 min. after injection of 25 mg. of 3:5-dinitro-*o*-cresol/kg. body wt., which had produced an increase of 4·3° in the colon temperature.

The oxygen uptake of these mitochondria in the presence of apyrase, the recovery of mitochondrial protein and their adenosine triphosphatase activity in the presence of 2:4-dinitrophenol were unaffected. However, the oxygen uptake without added apyrase and the adenosine triphosphatase activity without 2:4-dinitrophenol were much increased in the mitochondria from the treated rats (Tables 1 and 2).

In Expt. 13 the concentration of 3:5-dinitro-*o*-cresol in the isolated mitochondrial suspension was found to be 9 μM. The relationship between the concentration of 3:5-dinitro-*o*-cresol and mitochondrial adenosine triphosphatase activity *in vitro* has been determined; an activity of 3 μg.-atoms of P liberated/mg. of protein/hr. would require a concentration of 8 μM-3:5-dinitro-*o*-cresol in the mitochondrial suspension.

DISCUSSION

No differences were found in the behaviour of the mitochondria from the control rats exposed for short periods to widely differing environmental temperatures. It might be that the differences in metabolic rate thus produced were too small to cause detectable differences in the isolated mitochondria. Much greater metabolic changes occurred in the rats subjected to limb ischaemia, but there was no correlation between any of the properties measured on the isolated mitochondria and the colon temperature. Gross changes in the colon (liver) temperature in the range 22–40° have not produced comparable changes in the ability of the mitochondria to oxidize pyruvate, in the degree of stimulation on the addition of apyrase or in their adenosine triphosphatase activity both with and without 2:4-dinitrophenol.

The normal oxygen uptake of the mitochondria of rat liver *in vivo* cannot be assessed but it may be assumed that, after a lethal dose of 3:5-dinitro-*o*-cresol, when the effects are maximal mitochondrial oxidation is proceeding *in vivo* at its maximal rate (q_{O_2} around 100). The colon temperature at this time is 41–42°. With this as a reference point a temperature of 22° (Expt. 1) must clearly indicate that the mitochondria are working at a much lower rate; assuming a Q_{10} of 2 their oxidative rate would be 25 % of the maximum. The results in this paper do not suggest that this lowered activity is due to any intrinsic inability of the mitochondria to oxidize substrates of the tricarboxylic acid cycle.

When the results in the controls and in the rats after limb ischaemia are collected into two groups, certain differences in the mitochondria appear (Table 3). The oxygen uptake of the mitochondria from the experimental rats both with and without apyrase is significantly different from the controls but the degree of stimulation is not. These slight differences, which for adenosine triphosphatase activity are near the limit of resolution of the method, may be associated with a change in the composition of the liver after limb ischaemia, e.g. loss of glycogen (cf. Stoner, 1958*b*), altering the physical state of the homogenate and producing slight differences in the sedimentation of the particles. The differences in oxygen uptake and in adenosine triphosphatase activity could well be explained if the experimental mitochondrial preparation was less contaminated by other material.

The biochemical properties of the mitochondria as measured by our assay procedures may bear little quantitative relation to their behaviour *in vivo*. The ability of the mitochondria to respond to demands for ATP would seem to be an essential physiological mechanism, and the results in Expts. 12 and 13 have shown that 3:5-dinitro-*o*-cresol has a similar effect on mitochondrial adenosine triphosphatase activity whether added *in vitro* or *in vivo*. These results are not in conflict with those of Parker (1956) since in his experiments with 2:4-dinitrophenol the mitochondria were isolated at a much later stage, when the rats had recovered from the immediate effects of the compound. If our previous assumption is correct that oxygen uptake (and therefore adenosine triphosphatase activity, cf. Parker, 1958) is maximal *in vivo* after a lethal dose of 3:5-dinitro-*o*-cresol, then the much lower values obtained after isolation might be due to loss of 3:5-dinitro-*o*-cresol during the isolation procedures or to dilution during the assay. However, assuming the relation between mitochondrial protein and water found by Werkheiser & Bartley (1957), an overall concentration of 9 μM in the mitochondrial suspension indicates a concentration of approx. 200 μM in the mitochondria. Full understanding of these findings must await

more information on the distribution of 3:5-dinitro-*o*-cresol between mitochondria and medium under a variety of conditions.

Two ways in which mitochondrial metabolism could be influenced to give the lowered metabolism of the injured rats in Expts. 1–4 are structural damage to the mitochondria and alterations in the intracellular medium.

The first possibility seems very unlikely, for it would be necessary for this damage to be repaired during the isolation. Damage of this type would probably result in the loss of essential coenzymes and replacement of these coenzymes is not possible in these experiments since they have been carried out without further additions to the mitochondria.

Alterations in the concentrations of the normal intracellular constituents such as electrolytes would have to be very large to produce mitochondrial changes of the magnitude required, but the reactions leading to the formation of the substrates for the mitochondria might be more sensitive to such changes. The presence of an inhibitor in the intracellular fluid is a further possibility. This could be a direct inhibitor of the enzymes of the mitochondria or it could inhibit any enzyme leading to the formation of the substrates necessary for the mitochondria. There is evidence that the liver temperature in normal rats is depressed when mitochondrial oxidation is inhibited, as by fluoroacetate (Stoner, 1956), or when glycolysis is inhibited, as by D-2-deoxyglucose (Wick, Drury, Nakada & Wolfe, 1957; Stoner & Threlfall, 1959). In favour of the idea that the temperature fall in the early part of the response to limb ischaemia at normal environmental temperatures is related to a failure to form substrate (pyruvate) for the mitochondria is the accumulation of hexose diphosphate in the liver under these conditions (Stoner & Threlfall, 1959).

The rise in liver temperature after limb ischaemia when the environmental temperature is raised to 30° appears to be a local phenomenon (Stoner, 1958*a*). It is not accompanied by a general rise in metabolic rate, for the total oxygen consumption of the rat falls in the same way as in rats injured at a lower environmental temperature (20°). At the higher environmental temperature (30°) there is a fall in the ATP content of the liver (Stoner & Threlfall, 1954). As pointed out above, the attempt to repair this loss may stimulate metabolism and lead to increased heat production.

Workers on the mitochondria of other tissues have claimed that the properties of the mitochondria are changed in shock after various injuries. Packer *et al.* (1958) claim that oxidative phosphorylation by rat-heart and -brain mitochondria is decreased after limb ischaemia. The statistical evaluation of their published data is not possible. Others claim to have found decreased oxidative phosphorylation by heart mitochondria in haemorrhagic shock (Strawitz & Hift, 1956*a*, *b*; Hift & Strawitz, 1958). The significance of their results depends on one extremely low P/O ratio out of seven experiments. It has been the experience of this Laboratory that it is very difficult to obtain P/O ratios with a low standard error and it is our view that significant changes in the mitochondria isolated from animals subjected to physical injury have yet to be demonstrated. Much more work is necessary before the acute changes in tissue temperature in these pathological states are understood.

SUMMARY

1. Different liver temperatures have been produced in rats by varying the environmental temperature in ischaemic shock and by subcutaneous injection of 3:5-dinitro-*o*-cresol.

2. No correlation has been found between the properties of the liver mitochondria isolated from rats in ischaemic shock and their colon temperatures (range 22·0–40·5°). Possible reasons for these temperature changes are discussed.

3. The behaviour of the isolated mitochondria is explained by their 3:5-dinitro-*o*-cresol content. The relation between this and the rise in temperature *in vivo* is discussed.

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The Metabolic Fate of the Ester Sulphate Group of Potassium *p*-Nitrophenyl [^{35}S]Sulphate

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The fate of arylsulphates in the animal body has been investigated by a number of workers. Early studies by Rhode (1923), Sperber (1948) and Garton & Williams (1949) suggested that almost the whole of the administered dose of potassium phenyl sulphate was rapidly eliminated unchanged in the urine. Hawkins & Young (1954), using radioactive techniques, observed only slight hydrolysis (4–12%) of orally administered and intraperitoneally injected phenyl [^{35}S]sulphate and naphthyl [^{35}S]-sulphate. In contrast, the results of Hanahan & Everett (1950) indicate that the bulk of the dose of oestrone [^{35}S]sulphate is desulphated by adult female rats, and there is indirect evidence which suggests that appreciable hydrolysis of stilboestrol disulphate (Bishop, Richards & Perry, 1951) and of sulphated insulin (containing sulphated tyrosine residues, see Glendening, Greenberg & Fraenkel-Conrat, 1947) occurs *in vivo*. Michel, Roche, Closon & Michel (1958) noted that ^{131}I-labelled 3:5:3′-tri-iodo-L-thyronine sulphate is partially desulphated *in vivo* by the rat and eliminated as the corresponding glucosiduronic acid derivative. At first sight these collected results might suggest that only the sulphate esters of phenols which can be regarded as 'physiologically active' are extensively hydrolysed *in vivo*.

Mammalian tissues contain at least three distinct enzymes which are capable of hydrolysing arylsulphates. The specificity of these enzymes is not yet clearly defined, but there are indications that two of them, arylsulphatases A and B, show high affinity and activity towards arylsulphates such as dipotassium 2-hydroxy-5-nitrophenyl sulphate and monopotassium 4-chlorocatechol monosulphate (Dodgson & Spencer, 1957; Dodgson & Wynn, 1958), but comparatively low activity towards simpler arylsulphates such as potassium *p*-acetylphenyl sulphate and potassium *p*-nitrophenyl sulphate (cf. Dodgson, Rose & Tudball, 1959). In contrast, the third enzyme, arylsulphatase C, is particularly active towards these simpler arylsulphates. In spite of these differing specificities, all three enzymes show only comparatively feeble activity and affinity towards phenyl sulphate (Dodgson *et al.* 1959), and this can probably be attributed to the absence of an *ortho* or *para* electrophilic substituent group in the substrate molecule (cf. Dodgson, Spencer & Williams, 1956). It is perhaps not surprising therefore that phenyl sulphate is not extensively hydrolysed *in vivo*. Similar considerations probably apply to naphthyl sulphate.

In attempts to elucidate the physiological role of the arylsulphatase enzymes, recent work in these Laboratories has been directed towards a study of the ability of the sulphate esters of 'physiologically active' phenols to act as substrates for the various arylsulphatases both *in vivo* and *in vitro*. In many cases, the chemical synthesis of radioactive (^{35}S) specimens of these particular sulphate esters presents formidable problems. It was therefore considered desirable, in order to gain experience with various techniques, to conduct preliminary investigations with potassium *p*-nitrophenyl [^{35}S]sulphate, an arylsulphate which is readily prepared on a small scale and which, from theoretical considerations, might be expected to undergo appreciable desulphation *in vivo*.

The results of this preliminary study are presented in this paper.

MATERIALS AND METHODS

Potassium p-*nitrophenyl* [^{35}S]*sulphate*. This was prepared by a modification of the method of Burkhardt & Lapworth (1926). Carrier-free chloro[^{35}S]sulphonic acid (0·261 g., specific activity 10·4 mc/m-mole) was mixed with 0·28 g. of

unlabelled chlorosulphonic acid at 0°. To this was added 2 ml. of carbon disulphide followed by 1·72 ml. of diethylaniline, care being taken that the temperature did not rise above 5° during the addition. The temperature was then allowed to rise to that of the room, when 0·59 g. of *p*-nitrophenol suspended in 2 ml. of carbon disulphide was added with stirring. After stirring intermittently over a period of 2 hr. the whole was cooled in an ice–salt mixture, when the supernatant carbon disulphide could be poured off. Sufficient 10% KOH was added to make the residual syrup slightly alkaline, care being taken to keep the temperature below 35°. Water (10 ml.) was added to the alkaline syrup and the whole was extracted with three 20 ml. portions of ether to remove excess of diethylaniline. The cooled aqueous residue was adjusted to approx. pH 3 with N-H_2SO_4 before removal of excess of *p*-nitrophenol by extraction with three 20 ml. portions of ether. The pH of the aqueous residue was raised to about 11 with 10% KOH, and the whole poured into 4 vol. of acetone at 0°. The precipitated $K_2{}^{35}SO_4$ was kept at 0° for 1 hr. and removed by centrifuging; the supernatant was concentrated *in vacuo* at 37°. The residue was recrystallized twice from 90% ethanol and dried *in vacuo* at 0°. The yield of potassium *p*-nitrophenyl [^{35}S]sulphate (NP^{35}S) was 0·75 g. The radioactivity of the preparation was of the order of 15·2 μc/mg. Descending paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (50:12:25) showed the material to be homogeneous and free from inorganic [^{35}S]sulphate.

Experimental animals. Mature M.R.C. hooded rats were used throughout. These animals were injected intraperitoneally under light ether anaesthesia and were subsequently placed in metabolism cages designed to permit separate collection of urine and faeces. The animals were allowed water without restriction and were removed from the cages for 30 min. in each 24 hr. period, when they were permitted to feed. Any urine and faeces voided during this period were rejected. Urine and faeces were collected every 24 hr., the cages being washed down with 150 ml. of water. The urine and washings were pooled, filtered through a plug of cotton wool and, whenever possible, were used immediately; otherwise they were stored at 2° until required.

Measurement of radioactivity of fractions of urine. The inorganic sulphate fraction of the urine was precipitated as $Ba^{35}SO_4$ by a modification of the method of Folin (1905). A portion of the urine (15 ml.) was transferred to a tared, specially designed (see Walkenstein & Knebel, 1957) 50 ml. centrifuge tube and a known amount of carrier M-K_2SO_4 solution was added to give a final precipitate weight of approx. 100 mg. of $Ba^{35}SO_4$. A portion (4 ml.) of 2·5 N-HCl was then added, followed by 4 ml. of aqueous 10% $BaCl_2$, added dropwise. The tube was capped with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2) and allowed to stand without disturbance at room temperature for 16–20 hr. The precipitated $Ba^{35}SO_4$ was separated by centrifuging and washed three times by suspending it in 40 ml. portions of water, the suspension being kept for 1 hr., before each re-centrifuging. The precipitate was finally washed with 40 ml. of acetone and dried at 110° before weighing. Preliminary experiments showed that NP^{35}S was not hydrolysed during this procedure nor did it interfere in any way.

The precipitation of the combined inorganic and ester sulphate fraction of urine was carried out as described above, after hydrolysis of the urine (15 ml.) with 5 ml. of 2·5 N-HCl for 90 min. at the temperature of the steam bath. Preliminary experiments showed that no significant hydrolysis of NP^{35}S occurred during the period between the voiding of the urine and the analysis thereof.

The total sulphur fraction was also precipitated as $Ba^{35}SO_4$, after oxidation of the urine with fuming HNO_3 (sp.gr. 1·5) according to the method of Young, Edson & McCarter (1949).

The precipitated $Ba^{35}SO_4$ was plated for counting by a technique similar to that described by Eldjarn & Nygaard (1954). The amount of $Ba^{35}SO_4$ present in each planchet was determined by weighing the latter before and after plating. Radioactivity measurements were made with a thin end-window Geiger–Müller tube. Corrections for background, self-absorption, decay and coincidence were made and sufficient counts were recorded to give a standard error of less than 2%.

Measurement of ^{35}S content of faeces and tissues. The total sulphur content of faeces, individual tissues or of the whole rat was precipitated and counted as $Ba^{35}SO_4$ after oxidation with fuming HNO_3 according to the method of Young *et al.* (1949).

Detection of radioactivity on paper chromatograms. Radioactive spots were located on chromatograms with the aid of the C. 100 Actigraph automatic chromatogram-strip scanner (Nuclear-Chicago Corp., Ill., U.S.A.). Alternatively, radioautograms were obtained with Ilford Industrial B X-ray film. The exposure time was usually 21 days.

EXPERIMENTAL AND RESULTS

Rats were injected intraperitoneally with 5 or 40 μmoles of NP^{35}S in 0·5 ml. of water. In initial experiments, urine and faeces were collected every 24 hr. for a total of 96 hr. However, the amount of ^{35}S appearing in urine and faeces during the second half of this period was insignificant (less than 0·7% of the injected dose) and in subsequent experiments urine and faeces were collected during 48 hr. only. After 48 hr. the animals were killed by a blow on the back of the head and the whole carcass was taken for the determination of residual ^{35}S. Table 1 records the results. The faecal ^{35}S values are not included since, for the whole 48 hr. period, these were always less than 0·6% of the injected dose.

With male rats given the lower dose approx. 90% of the radioactivity of the dose was recovered from urine, faeces and carcass. A substantial amount (25%) of the initial dose of ^{35}S was present as inorganic sulphate. With the higher dose the recoveries of ^{35}S were rather low (80%) and the degree of hydrolysis of NP^{35}S was somewhat reduced. With the female rats the recovery of ^{35}S was complete, with the inorganic [^{35}S]sulphate accounting for 29·5% of the initial dose of NP^{35}S. No great significance can be attached to the variations between the three groups of animals in view of the small number of animals tested. It is, however, clear that appreciable hydrolysis of NP^{35}S occurs *in vivo*.

Retention of ^{35}S in tissues

Two male rats were injected with 5 μmoles of NP^{35}S and after 48 hr. they were killed. The individual tissues from each rat were combined, washed with water, dried between

Table 1. *Distribution of* ^{35}S *in the urine and carcass of rats injected intraperitoneally with potassium* p-*nitrophenyl* [^{35}S]*sulphate*

Results are average values with the range in parentheses. ^{35}S was precipitated and counted as $Ba^{35}SO_4$ by the procedures described in the text. The radioactivity of the respective doses was $6{\cdot}2 \times 10^5$ counts/min. (5 μmoles) and 5×10^6 counts/min. (40 μmoles), measured after hydrolysis as an infinitely thick plate of $Ba^{35}SO_4$.

Sex	Dose (μmoles)	No. of animals	Range of body wt. (g.)	Distribution of ^{35}S in urine (% of ^{35}S injected)						Residual ^{35}S in carcass after 48 hr. (% of ^{35}S injected)
				Inorganic sulphate fraction		Combined inorganic and ester sulphate fraction		Total urinary sulphur		
				24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.	
Male	5	7	300–400	22·8 (15·4–30·2)	1·5 (1·1–2·0)	81·2 (74·9–89·6)	1·6 (1·3–1·8)	83·2 (76·9–91·6)	3·0 (2·8–3·5)	3·5 (2·8–4·1)*
Male	40	3	220–280	17·5 (13·4–22·2)	1·6 (1·3–2·3)	70·8 (68·4–71·3)	2·4 (1·7–3·2)	72·6 (70·2–73·1)	3·6 (2·5–5·5)	3·0 (1·4–5·5)
Female	5	3	250–310	26·7 (15·9–32·6)	2·8 (1·7–3·1)	99·0 (98·3–99·6)	3·0 (2·1–4·0)	99·5 (99·0–99·5)	3·2 (2·5–4·1)	2·5 (1·8–3·0)

* Average of four rats.

filter paper, weighed and dissolved in a known volume of fuming HNO_3. The ^{35}S content of suitable volumes (usually 1 ml.) was precipitated as $Ba^{35}SO_4$ after oxidation. Blood was collected by heart puncture and treated in a similar fashion. Table 2 shows that all the tissues examined retained some ^{35}S. Almost the whole of the radioactivity of blood was associated with the plasma. Skin and cartilage also retained ^{35}S (see below).

Appearance of ^{35}S *in skin, cartilage and liver*

Since injected inorganic sulphate is incorporated into the sulphated mucopolysaccharides of connective tissue (e.g. Dziewiatkowski, 1951; Boström, 1952) and into the liver taurine (Boström & Åqvist, 1952), it was investigated whether a similar uptake of [^{35}S]sulphate occurred after administration of $NP^{35}S$. Six rats (four male and two female) each received injections of 5 and, 2 hr. later, 10 mg. of $NP^{35}S$. The rats were kept in pairs in metabolism cages for 24 hr. and the urine was collected for chromatographic experiments. The rats were then killed and the skins, livers and sternal, xiphisternal and tracheal cartilage were pooled.

Treatment of skin and cartilage. All hair was removed with a razor before maceration of the skin in acetone at 0°. The defatted material was then dried in air (total wt. from six rats was 53·2 g.). Cartilage was also macerated in acetone at 0° and dried (total wt. 1·53 g.). The dried skin or cartilage was extracted by mechanical shaking for 24 hr. at 27° with 0·5 N-NaOH (15 ml./g. of material). The cloudy supernatant separated by centrifuging for 30 min. at 5000 *g* was stored at 0° while the residue was re-extracted as before with 0·5 N-NaOH (5 ml./g. of original dried material) and centrifuged. The extracts were combined, toluene was added as a preservative and the whole was dialysed against running tap water for 48 hr., followed by distilled water for 48 hr. at 4°. The pH of the extract was adjusted to 7·8 with N-NaOH before incubation with papain (L. Light and Co. Ltd.; 10 mg./g. of original dried material) at 37° for 5 days. The incubation was carried out in dialysis tubing immersed in 0·1 M-NaH_2PO_4 which had been adjusted to pH 7·8 with N-NaOH. After incubation, one-third of the volume of 40% trichloroacetic acid was added and precipitated material separated by centrifuging. The supernatant was dialysed for 48 hr. against several changes of distilled water at 4° before being concentrated

Table 2. *Retention of* ^{35}S *by rat tissues* 48 *hr. after intraperitoneal injection of potassium* p-*nitrophenyl* [^{35}S]*sulphate*

Each rat received 5 μmoles of $NP^{35}S$, the radioactivity of the dose being $6{\cdot}2 \times 10^5$ counts/min., measured after hydrolysis as an infinitely thick plate of $Ba^{35}SO_4$.

Tissue	Total wt. (from two rats)	^{35}S content (counts/min./g. wet wt. of tissue)
Spleen	2·1	110
Pancreas	1·9	110
Kidney	5·4	160
Liver	25·1	100
Heart and aorta	2·3	250
Blood	—	58*

* Counts/min./ml.

to one-tenth of its volume *in vacuo* at 37°. The concentrated extract was stirred for 30 min. with potassium acetate, acetic acid and kaolin (1 g., 1 ml. and 1 g. respectively/50 ml. of concentrate). The supernatant separated by centrifuging at 5000 *g* for 15 min. was kept at 0° while the residue was washed three times by suspending it in 50 ml. portions of water and re-centrifuging. All the supernatants were combined and concentrated to low bulk *in vacuo* at 37°. Ethanol (4 vol. of 95%, v/v) was added and the whole kept overnight at 0°. The precipitated material was separated by centrifuging and washed well with ethanol followed by ether. The material was dried *in vacuo* at 2° over P_2O_5. The yield of crude mucopolysaccharide from skin was 785 mg., and from cartilage, 73 mg.

The crude mucopolysaccharide was further purified by paper electrophoresis. The cartilage preparation (34 mg.) and that from skin (120 mg.) were dissolved in 0·1 M-ammonium acetate–acetic acid buffer, pH 6·0 (5 μl./mg. of mucopolysaccharide) and subjected to horizontal paper electrophoresis in the same buffer, on Whatman no. 17 paper for 16 hr. at a potential gradient of 6 v/cm. Control preparations of bovine cartilage chondroitin sulphate (Dodgson, Lloyd & Spencer, 1957) and hyaluronic acid (Wyeth, Philadelphia, Pa., U.S.A.) were run simultaneously. Acid mucopolysaccharides were detected by pressing a strip of Whatman no. 1 paper on the wet no. 17 paper at the end of the run and subsequently staining the 'print' with the Alcian blue technique of Heremans & Vaerman (1958). With the skin preparation two staining zones corresponding to hyaluronic acid and chondroitin sulphate were detected, whereas with the cartilage preparation only the chondroitin sulphate zone was observed. The appropriate areas of the electrophoresis strip were then eluted with water and the eluates concentrated to a syrup *in vacuo* at 37°. Acid mucopolysaccharide was then precipitated by the addition of 50 ml. of 95% ethanol containing 0·5 g. of potassium acetate. The precipitated material was separated by filtration through weighed filter-paper disks. The disks were subsequently dried, re-weighed and counted for radioactivity. The hyaluronic acid fraction from skin, as expected, contained no radioactivity. The chondroitin sulphate fraction from skin showed 36 counts/min./mg. and that from cartilage 146.

Treatment of liver. Taurine was isolated from the liver by a procedure based on that of Boström & Åqvist (1952). The livers (64·6 g.) were macerated in 200 ml. of ethanol. The whole was filtered, the filtrate being retained and the residue was then re-extracted with a further 100 ml. of ethanol. The pooled extracts were stored at 4° whilst the residue was refluxed for 2 hr. with ethanol–ether (3:1, v/v). All extracts were combined and concentrated to dryness *in vacuo*. The concentrate was dissolved in the smallest possible volume of $CHCl_3$ and re-precipitated by pouring into a large excess of acetone. The precipitated material was separated, re-dissolved in $CHCl_3$ and re-precipitated with acetone. The precipitate was separated and hydrolysed by boiling under reflux with 100 ml. of 6 N-HCl for 24 hr. After cooling, the solidified fatty acids were removed and the solution was concentrated to dryness *in vacuo* at 37°. The residue was dissolved in water and then re-concentrated as before to remove excess of HCl. This procedure was repeated several times. The residue was finally dissolved in 4 ml. of water and a portion (2 ml.) was applied to a column (15 cm. × 1 cm.) of Dowex-50 ion-exchange resin (H^+ form, × 8, mesh 200–400) which had been equilibrated with 0·1 M-sodium citrate–HCl buffer, pH 3·41, as described by Moore & Stein (1951). The column was eluted with the same buffer and the first 50 ml. of eluate was collected in 25 equal fractions. Horizontal paper electrophoresis on Whatman no. 1 paper in 0·1 M-ammonium acetate–acetic acid buffer, pH 4·5, with a potential gradient of 12 v/cm. for 2 hr., showed that fractions 10–15 contained one ninhydrin-positive component only. The electrophoretic mobility of the component was identical with that of an authentic sample of taurine. These fractions were pooled, concentrated to dryness and the residue was dissolved in the minimum volume of water and subjected to paper electrophoresis as described above but with Whatman no. 17 paper. The taurine was located on a 'print' by spraying with ninhydrin and the appropriate zone was eluted with water. The concentrated elute was counted for radioactivity. The total yield (from 64·6 g. of liver) gave 312 counts/min. No quantitative significance could be attached to the count since the material was contaminated with appreciable amounts of the citrate buffer which was used to elute the taurine from the ion-exchange column.

Chromatographic behaviour of ^{35}S-labelled compounds in urine

The undiluted urine of male and female rats which had each received 15 mg. of NP^{35}S as described above was subjected to paper chromatography on Whatman no. 1 paper with butan-1-ol–acetic acid–water (50:12:25) with downward irrigation. Where practical, sufficient urine was applied to the paper to give approx. 1000 counts/min. (measured on the paper with a Panax monitor). Urines from animals which had received lower doses (1·28 mg.) of NP^{35}S were treated in a similar fashion. In addition, urines from male and female rats which had each been injected with a mixture of 0·1 ml. of a carrier-free solution of $Na_2{}^{35}SO_4$ (0·5 mc) and 0·4 ml. of 0·15 M-Na_2SO_4 solution, were examined simultaneously. Chromatograms were run for 16 hr. at room temperature and dried at the same temperature before being examined for radioactivity. Preliminary experiments in which freshly voided normal rat urine (15 ml.) was kept with 15 mg. of NP^{35}S at room temperature for 24 hr. before subjecting it to paper chromatography showed that NP^{35}S was stable under these conditions and that no exchange of sulphate occurred between the compound and other sulphate esters present in the urine.

Table 3 records the chromatographic mobilities (in relation to that of NP^{35}S) of the various radioactive spots which were detected on chromatograms of the urine of female rats receiving either $Na_2{}^{35}SO_4$ or NP^{35}S. After administration of $Na_2{}^{35}SO_4$, 11 radioactive spots were obtained in addition to inorganic [^{35}S]sulphate. After administration of NP^{35}S, ten radioactive spots in addition to inorganic [^{35}S]sulphate and NP^{35}S were detected. Spot 7 was not present on chromatograms of the $Na_2{}^{35}SO_4$ urines and had the same chromatographic mobility as that of an authentic specimen of *p*-aminophenyl sulphate, a possible metabolite of NP^{35}S. Other differences observed were the absence of spots 2 and 13 from the NP^{35}S urine and the absence of spot 11 (possibly a second metabolite of NP^{35}S) from the $Na_2{}^{35}SO_4$ urines. Spot 15 could not be detected in the NP^{35}S urine because of the overlap of the very dense

Table 3. *Chromatographic mobilities in butan-1-ol–acetic acid–water* (50:12:25) *of radioactive compounds present in the urine of female rats receiving potassium* p-*nitrophenyl* [^{35}S]*sulphate or* $Na_2{}^{35}SO_4$

Experimental details are given in the text. The mobilities quoted are average values obtained from several different experiments and are expressed (R_{NPS}) relative to that of NP^{35}S, which is taken as unity.

	R_{NPS}	
Spot no.	$Na_2{}^{35}SO_4$ urine	NP^{35}S urine
1 ($Na_2{}^{35}SO_4$)	0·12	0·12
2	0·19	—
3	0·23	0·23
4	0·33	0·33
5	0·41	0·41
6	0·45	0·45
7	—	0·55
8	0·59	0·59
9	0·65	0·65
10	0·71	0·71
11	—	0·75
12	0·83	0·83
13	0·92	—
14 (NP^{35}S)	—	1·00
15	1·04	?

spot associated with NP^{35}S. The chromatographic pattern of the urine of male rats receiving $Na_2{}^{35}SO_4$ was virtually identical with that of female rats. On the other hand, with the NP^{35}S urines of male rats only spots 1, 3, 6, 7, 8, 10, 12 and 14 (see Table 3) could be detected with certainty.

Similar chromatographic experiments were run with NP^{35}S and $Na_2{}^{35}SO_4$ urines which had been hydrolysed at 95° with N-HCl for 90 min. or, alternatively, which had been incubated for 4 hr. at 37° and pH 6·5 with the digestive juice of *Helix pomatia* (the richest known source of arylsulphatase and a potent source of steroid sulphatase; see Henry & Thevenet, 1952). In these cases, only inorganic [^{35}S]sulphate could be detected on chromatograms, indicating that the radioactive compounds present in the unhydrolysed urines were all ester sulphates (either aryl- or 3β-sulphates of 5α- and Δ^5-steroids).

DISCUSSION

The results obtained provide strong evidence that hydrolysis of arylsulphates by the arylsulphatase enzymes does occur *in vivo*. Alternative mechanisms whereby inorganic sulphate could arise from NP^{35}S are, of course, possible. For example, excretion of NP^{35}S via the bile could be followed by degradation of the ester by the bacterial flora of the gut with subsequent reabsorption of inorganic sulphate from the large intestine. A similar effect has been observed in rats by Dohlman (1956) after the injection of ^{35}S-labelled chondroitin sulphate. However, it is doubtful whether a biliary circulation of this sort could account for the amounts of inorganic [^{35}S]sulphate which were found in the 24 hr. urines in the present work. The negligible amounts of radioactivity which were observed in faeces also support this conclusion. It is perhaps significant that Hawkins & Young (1954) failed to obtain any marked increase in the output of inorganic [^{35}S]sulphate in the urine when phenyl [^{35}S]sulphate was administered orally rather than by intraperitoneal injection.

If it is accepted that arylsulphatase activity is mainly responsible for the production of inorganic sulphate from NP^{35}S then it can tentatively be assumed, from specificity studies (see Dodgson *et al.* 1959), that arylsulphatase C is the responsible enzyme. Moreover, the fact that the degree of hydrolysis observed in the present work is greatly in excess of that noted for phenyl [^{35}S]sulphate (4% in 4 days) by Hawkins & Young (1954), suggests that the relative specificity of the enzyme in the living organism is similar to that shown *in vitro*.

The complex pattern of radioactivity in chromatograms of the urines of rats receiving $Na_2{}^{35}SO_4$ (see also Boström & Vestermark, 1959) or NP^{35}S is of interest. No attempt has yet been made to identify the unknown components but it seems clear that they are either aryl or steroid sulphate esters. For animals injected with $Na_2{}^{35}SO_4$ it is virtually certain from the work of Lipmann and others (see Dodgson, 1958) that these sulphate esters arise from the inorganic sulphate through the intermediate formation of 3′-phosphoadenosine 5′-phosphosulphate ('active sulphate'), and the subsequent transfer of sulphate from this compound under the influence of the appropriate sulphokinase. With animals receiving NP^{35}S, the urine ester [^{35}S]sulphate pattern is, in most respects, similar to that of the urine of animals receiving $Na_2{}^{35}SO_4$. There are two theoretically possible metabolic routes whereby the sulphate group of NP^{35}S could be made available to other phenols or steroids. Hydrolysis of NP^{35}S by arylsulphatase enzymes would provide inorganic sulphate which could subsequently be used for the sulphation of the appropriate phenolic and steroid acceptors by the usual 3′-phosphoadenosine 5′-phosphosulphate synthetic route. However, Gregory & Lipmann (1957) have shown that, in the presence of 3′:5′-diphosphoadenosine, liver phenolsulphokinase can also catalyse the direct transfer of sulphate from NPS to suitable phenolic acceptors, provided that the product has a sulphate group potential well below that of NPS. 3′:5′-Diphosphoadenosine acts as a coenzyme for this transfer. This direct type of mechanism may be contributing significantly to the production of some of the radioactive arylsulphate esters which were present in the NP^{35}S urines.

Whether, in the presence of the appropriate sulphokinases, NP^{35}S can transfer sulphate, via 3′:5′-diphosphoadenosine, to non-phenolic acceptors is

still not known. Attempts by Gregory & Lipmann (1957) to test this possibility with enzyme preparations containing both phenol and steroid sulphokinases were unsuccessful. In view of this it is also wise to assume, for the time being, that the ^{35}S which appears in skin and cartilage chondroitin sulphate after injection of NP^{35}S is not incorporated by direct transfer from the latter. More probably, inorganic sulphate derived from NP^{35}S by action of arylsulphatase is the starting material for this incorporation (see D'Abramo & Lipmann, 1957).

It is possible, of course, that the arylsulphatase enzymes might themselves be capable of transferring sulphate directly from NP^{35}S to a variety of acceptors. Attempts have been made in these Laboratories (unpublished) to transfer sulphate from NPS or dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate) to alcohols, phenols, simple sugars, amino sugars, *N*-acetylchondrosin and chondroitin, with purified arylsulphatases of mammalian and molluscan origin. No evidence of transfer has yet been obtained.

The occurrence of small amounts of ^{35}S in rat-liver taurine after the administration of NP^{35}S provides some support for the view that inorganic sulphate sulphur can give rise to taurine sulphur in the intact rat (Boström & Åqvist, 1952). A similar utilization of inorganic sulphate for taurine synthesis has been noted in the developing chick embryo (Machlin, Pearson & Denton, 1955; Lowe & Roberts, 1955). The process does not appear to involve the preliminary formation of sulphur-containing amino acids and the biosynthetic route involved is at present obscure.

SUMMARY

1. After intraperitoneal injection of potassium *p*-nitrophenyl [^{35}S]sulphate to male and female rats, the bulk of the ^{35}S appears in the urine within 24 hr.

2. Up to 30% of the ^{35}S of the original dose appears in the urine as inorganic [^{35}S]sulphate, most of the remainder appearing as ester [^{35}S]-sulphate.

3. Chromatographic examination of the urines shows the presence of at least ten radioactive sulphate esters in addition to potassium *p*-nitrophenyl [^{35}S]sulphate. The chromatographic pattern is very similar to that obtained with the urines of rats receiving $Na_2{}^{35}SO_4$ by intraperitoneal injection.

4. After 48 hr., ^{35}S is still present in the tissues. Incorporation of ^{35}S into the chondroitin sulphate of skin and cartilage and into liver taurine has been demonstrated.

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The Synthesis of Methionine by Suspensions of *Escherichia coli*

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The first indication that *p*-aminobenzoic acid is concerned in the synthesis of methionine by micro-organisms was the observation that methionine, under appropriate conditions, overcame inhibition of growth by sulphonamides in a non-competitive manner (Harris & Kohn, 1941; Kohn, 1943; Bliss & Long, 1941). Since then methionine has frequently been shown with various organisms to be an essential component of mixtures which replace *p*-aminobenzoic acid either in overcoming sulphonamide inhibition or in supporting growth of organisms requiring this vitamin (e.g. Lampen, Jones & Roepke, 1949; Strehler, 1950; Cutts & Rainbow, 1950). There is thus strong presumptive evidence that *p*-aminobenzoic acid has a function at one or more steps in methionine synthesis.

A role for cobalamin in the same synthesis was first found by Davis & Mingioli (1950), who isolated mutants of *Escherichia coli* requiring for growth either methionine or cobalamin; the relative concentrations required suggested that cobalamin might function as a catalyst in the synthesis of methionine.

Studies of the nutritional properties of mutant strains of bacteria and moulds requiring methionine for growth have provided strong presumptive evidence that this amino acid is derived from cysteine through the intermediary formation of cystathionine and homocysteine; the last step is thus the methylation of homocysteine (Horowitz, 1947; Teas, Horowitz & Fling, 1948; Lampen, Roepke & Jones, 1947; Gots & Koh, 1950; Gots, 1951; Plough, Miller & Berry, 1951). Since homocysteine will not replace methionine in the sparing of either *p*-aminobenzoic acid or cobalamin, it is likely that the function of both these vitamins is at the last, or methylation, step in the formation of methionine. The work of Emerson (1950) with *Neurospora* suggests that this stage may be complex.

The object of the present work was to obtain a simpler system for the synthesis of methionine than the whole growing culture, and one in which all the reactants could be clearly defined. It should then be possible to study with greater precision the roles of *p*-aminobenzoic acid and cobalamin, and to build up a background of knowledge from which to extend the work to isolated enzyme systems. At the present stage two points were of particular importance: (*a*) the nature of the ultimate source of the one-carbon-atom unit which gives rise to the methyl group of methionine; (*b*) the question whether the function of *p*-aminobenzoic acid is exerted after its conversion into a member of the folic acid group; there was no evidence on this point at the time the work was begun.

E. coli was chosen for the present work because (*a*) a number of auxotrophic mutants were available in which it was possible to control experimentally the presence or absence of a number of the metabolites in question, and (*b*) preliminary tests showed that suspensions did not destroy added methionine to any appreciable extent.

The results with whole organisms in washed suspensions are here reported in some detail since they form an essential background to later work with cell-free enzyme preparations. Part of the present work has been the subject of preliminary reports (Gibson & Woods, 1952; Gibson, 1952).

METHODS

Organisms and media

Escherichia coli. A number of strains (Table 1), many of them mutants requiring amino acids or vitamins for growth, were used. All were maintained by monthly subculture on tryptic digest of meat–agar slopes stored at 4° after incubation for 18 hr. at the required temperature (Table 1); they were stable during the period of the work.

The growth medium (*A*) for the preparation of suspensions of organisms was that of Davis & Mingioli (1950), except that the glucose was autoclaved with the other constituents. It was supplemented when necessary with the required growth factor at the concentrations given in Table 1. The supplement used for the growth of mutants requiring *p*-aminobenzoic acid in the partial or complete absence of that factor was (per litre of final medium): either (*a*) acid-hydrolysed casein (vitamin-free, prepared by the method of Snell & Rannefeld 1945), equivalent to 1·5 g. of original casein; thymine, 20 mg.; adenine, guanine and xanthine, each 5 mg. (supplement *E*); or (*b*) glycine, 20 mg.; DL-serine, DL-threonine, DL-lysine hydrochloride, DL-histidine hydrochloride and DL-tyrosine, each 40 mg.; DL-methionine, 10 mg.; thymine, 30 mg.; adenine, guanine, xanthine and uracil, each 5 mg. (supplement *F*). Both supplements were adjusted to pH 7·0.

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Table 1. *Strains of* Escherichia coli *used*

Strain no.*	Requirement for growth on medium *A*: Substance	Concn. used	Incubation temp.	Inoculum for bulk cultures†
273/384 (A.T.C.C. 9723a)	*p*-Aminobenzoic acid *or* Replacement mixture (see text)	μM See text	37° 37	0·1 ml.; 1 in 75
200 (from C 181)	Cobalamin *or* Methionine	1 μmg./ml. 0·13 mM (DL-)	30 30	0·1 ml.; 1 in 75
113/3	Cobalamin *or* Methionine	1 μmg./ml. 0·13 mM (DL-)	37 37	0·1 ml.; 1 in 75
PA 15	Serine *or* Glycine	10 mM (DL-) 10 mM	37 37	0·1 ml.; 1 in 10
55/5	Serine *or* Glycine *or* None	10 mM (DL-) 10 mM —	37 37 25	0·1 ml.; 1 in 10 0·1 ml.; 1 in 10 0·2 ml.; undiluted
518 (A.T.C.C. 9723)	None	—	37	0·1 ml.; 1 in 75
86 (N.C.T.C.)	None	—	37	0·1 ml.; 1 in 75

* Strains 200, 113/3 and 55/5 were from the collection of Dr B. D. Davis; strains 273/384 and 518 were provided by Dr R. Roblin and PA 15 by Dr Barbara Wright.

† Volume and dilution of inoculum development culture (see text) used to inseminate 100 ml. cultures for the preparation of suspensions of organisms.

Leuconostoc mesenteroides *P60*. This organism (American Type Culture Collection no. 7881) was used for the assay of methionine and glycine; it was maintained as described by Lascelles, Cross & Woods (1954). The basal medium was modified from that used by these authors for serine assay and contained (per litre of final medium): DL-alanine, 500 mg.; L-arginine hydrochloride, 200 mg.; DL-aspartic acid, 200 mg.; DL-asparagine, 800 mg.; L-cysteine hydrochloride, 130 mg.; DL-glutamic acid, 600 mg.; DL-histidine hydrochloride, 60 mg.; DL-isoleucine, 240 mg.; DL-leucine, 240 mg.; DL-lysine hydrochloride, 100 mg.; DL-phenylalanine, 100 mg.; DL-proline, 50 mg.; DL-serine, 210 mg.; DL-threonine, 60 mg.; DL-tryptophan, 40 mg.; DL-tyrosine, 200 mg.; DL-valine, 240 mg.; glucose, 25 g.; sodium acetate trihydrate, 20 g.; NH_4Cl, 3 g.; KH_2PO_4, 0·6 g.; $Na_2HPO_4,12H_2O$, 0·6 g.; $MgSO_4,7H_2O$, 0·4 g.; $MnSO_4,4H_2O$, 20 mg.; $FeSO_4,7H_2O$, 20 mg.; xanthine, 12 mg.; adenine, guanine and uracil, each 10 mg.; riboflavin, 0·5 mg.; nicotinic acid, 1 mg.; calcium pantothenate, 0·6 mg.; thiamine hydrochloride, 0·6 mg.; biotin, 1 μg.; *p*-aminobenzoic acid, 0·14 mg.; pH adjusted to 6·8. Pyridoxal (Seitz-filtered) was added with the inoculum to a final concentration of 0·2 μM. The basal medium was prepared as described for the medium *L* of Lascelles *et al.* (1954). Glycine (75 mg./l.) or DL-methionine (75 mg./l.) was added for the assay of methionine and glycine respectively.

Preparation of suspensions of Escherichia coli

Medium *A* (2 ml. in 125 mm. × 16 mm. tubes), containing the appropriate growth factor, was inoculated from a fresh slope culture and incubated for 24 hr. (48 hr. with *E. coli* 55/5) at the temperature shown in Table 1. Roux bottles containing the same medium (100 ml.) were sown with 0·1 ml. of a dilution of this culture (Table 1) chosen to yield growth equivalent to 0·05–0·1 mg. dry wt. of organisms/ml. after incubation for about 16 hr. in the horizontal position. The organisms were centrifuged, washed once on the centrifuge with half the culture volume of 6·7 mM-phosphate buffer, pH 7·4, and finally suspended in 0·133 M-buffer at twice the concentration desired in the solution used for testing methionine synthesis.

In some earlier experiments, the organisms were incubated (0·1 mg. dry wt./ml.) for 5 hr. at 37° in a mixture of glucose (0·01 M) and phosphate buffer (0·067 M; pH 7·4) before testing for methionine synthesis; they were then centrifuged and suspended as above. Such organisms will be referred to as 'preincubated'; under certain conditions they showed increased ability (variable and never more than twofold) to synthesize methionine compared with untreated organisms.

Assessment of growth and mass of organisms

The extent of growth of cultures and the dry weight of suspensions of organisms was assessed with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) in 6 mm. tubes and a neutral density filter. The relationship between galvanometer reading and dry weight was determined for each of the organisms used. With the different strains of *E. coli* the dry wt./ml. corresponding to a reading of 10 varied only from 0·29 to 0·36 mg.

Throughout this paper the quantity of organisms expressed as milligrams refers to the dry weight.

General procedure for study of methionine synthesis

Reaction mixture. The organisms were suspended at a concentration of 0·1–0·4 mg. dry wt./ml. in solution *M* (2·5 ml.) which contained basically: DL-homocysteine (6 mM), glucose (0·02 M) and phosphate buffer (0·067 M, pH 7·4). The tubes (125 mm. × 16 mm.) were incubated upright in air at 37° unless otherwise stated; anaerobic conditions did not improve methionine synthesis.

In each experiment there was a control in which the complete reaction mixture was heated for 5 min. at 100° at zero time.

Preparation of material for analysis. The reaction was stopped, and any free methionine within the organisms liberated, by heating to 100° for 10 min. Samples of the supernatant fluid after centrifuging were used for microbiological assay and chromatography.

In some experiments to confirm the identity of methionine the products were acid-hydrolysed or oxidized. For the former, the sample (10 ml.) was mixed with 10 N-HCl (10 ml.) and refluxed for 4 hr. Excess of acid was removed by evaporation to dryness under reduced pressure; this was twice repeated, 10 ml. of water being added between each distillation. The final residue was taken up in a little water, neutralized with 0·1 N-NaOH and diluted to the original volume. For oxidation, the sample (10 ml.) was treated with an equal volume of 30 % (w/v) H_2O_2 for 1 hr. at room temperature and the solution evaporated to dryness on a boiling-water bath; the destruction of H_2O_2 was completed by two further evaporations after adding water (20 ml.). The residue was dissolved in 10 ml. of water.

Microbiological assay of methionine and glycine

Leuconostoc mesenteroides *P 60.* This organism has been reported as suitable for the assay of methionine and glycine (Riesen, Schweigert & Elvehjem, 1946; Dunn, Camien, Shankman & Block, 1946). This was confirmed in the present work and a linear and reproducible growth response was obtained for quantities of both DL-methionine and glycine in the range 0·01–0·1 μmole (Fig. 1).

Procedure. The technical details were as described for the assay of serine with the same organism (Lascelles *et al.* 1954; Lascelles & Woods, 1954), except that (*a*) the modified media described above were used, (*b*) the organisms of the inoculum were washed once on the centrifuge with twice the culture volume of water and resuspended in the culture volume of water, (*c*) incubation was always in air and for 44–48 hr.

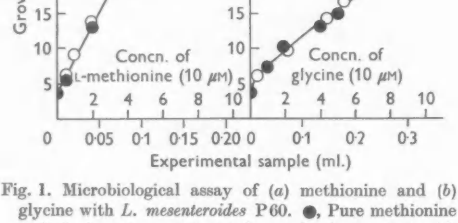

Fig. 1. Microbiological assay of (*a*) methionine and (*b*) glycine with *L. mesenteroides* P 60. ●, Pure methionine and glycine; ○, experimental samples from a test with suspensions of *E. coli* 55/5 incubated in solution *M* plus serine (10 mM) and cobalamin (10 μmg./ml.). Growth is recorded as EEL photoelectric colorimeter readings.

The samples under test (or appropriate dilutions) at two different concentrations were added in quantities of 0·1–0·8 ml.; standards containing three different quantities of DL-methionine (usually 0·01, 0·04 and 0·1 μmole) or glycine (0·02, 0·08 and 0·2 μmole) were set up in each complete assay.

Growth response to experimental samples. It was possible to match the dose/response curve for typical samples from experiments with suspensions of organisms to that for pure methionine or glycine over the whole of the useful assay range (Fig. 1). In methionine assays on samples containing less than 0·01 μmole/ml. (and which had therefore to be added undiluted to the assay tubes in up to 0·8 ml. amounts) the growth attained with larger amounts was occasionally less than would have been expected from the growth given by half the quantity; in such cases the higher value for methionine was accepted since it appeared that material inhibitory to the assay organism was present. Values for methionine of less than 0·2 μmole/mg. dry wt. of organisms in the terminology below were therefore subject to greater than the usual error.

Specificity. The organism responded only to the L-isomer of methionine; the racemic mixture was used as standard since the presence of D-isomer did not affect the results.

The samples under assay were likely to contain relatively high concentrations (with respect to biological activity) of components of solution *M* and experimental supplements. Various mixtures of homocysteine, serine, cobalamin, pteroylglutamic acid, N^5-formyltetrahydropteroylglutamic acid, choline and betaine neither replaced methionine for *Leuconostoc mesenteroides* P 60 nor affected growth in optimum methionine concentrations. The heated control (see above) also served as a check in this respect. Glycine was not replaced by glycollic acid or betaine.

Assessment of results. The methionine or glycine content of samples was obtained by direct comparison of the responses obtained at two concentrations with the dose/response curve given by the standards. Results are expressed as μmoles of L-methionine or glycine formed/mg. dry wt. of *E. coli*. Variation in replicate experiments, and the recovery values for methionine or glycine added to typical samples, showed that the over-all accuracy of the method was about ±10 %.

Microbiological assay of folic acid

Lactobacillus casei *factor.* The assay described by Lascelles & Woods (1952) was used. Results are expressed as concentration of the factor in the samples in terms of pteroylglutamic acid, which was used as standard.

Leuconostoc citrovorum *factor.* The details of the procedure given by Lascelles & Woods (1954) were followed; the standard was N^5-formyltetrahydropteroylglutamic acid (leucovorin) and results are expressed in terms of the equivalent concentration of this substance.

Chromatography

Paper. Descending chromatograms were developed on Whatman no. 1 paper with either (*a*) 'butan-1-ol–acetic acid' [the upper layer of butan-1-ol–acetic acid–water

Table 2. *Synthesis of methionine by suspensions deficient in* p-*aminobenzoic acid or cobalamin*

Organisms (0·1 mg./ml., strain 273/384; 0·18 mg./ml., strain 200) were incubated for 16 hr. and 8 hr. respectively in solution *M* modified as indicated. *p*-Aminobenzoic acid, μM; cobalamin, 10 μmg./ml. 'Preincubated' cells were incubated at 37° for 5 hr. in glucose (0·01 M) and phosphate buffer (0·067 M, pH 7·4) before testing for methionine synthesis.

Strain	Substances added (+) or omitted (−)				L-Methionine formed (μmoles/mg. of organisms)	
	DL-Homocysteine	Glucose	*p*-Aminobenzoate	Cobalamin	Untreated organisms	'Preincubated' organisms
273/384 (deficient in *p*-aminobenzoic acid)	+	+	+	−	.	0·35
	+	+	+	+	.	0·60
	+	+	−	+	.	<0·05
	+	−	+	+	.	0·07
	−	+	+	+	.	<0·05
200 (deficient in cobalamin)	+	+	−	+	0·60	0·86
	+	+	−	−	<0·05	<0·05
	+	−	−	+	<0·05	0·17
	−	+	−	+	<0·05	<0·05
	+	+	+	−	<0·05	.
	+	+	+	+	0·58	.

(4:1:5, by vol.)] or (*b*) 'phenol–water' (a saturated solution of water in A.R. phenol; the atmosphere of the tank was saturated with acetic acid vapour). The chromatograms were run until the solvent front had advanced 30–40 cm. (18–24 hr. at room temperature). The detecting agent was ninhydrin.

Resin. Columns of Dowex-50 resin were used for the separation of glycine from certain reaction mixtures. The procedure of Moore & Stein (1951) was used with the details given by Lascelles & Woods (1954).

Chemicals

DL-*Homocysteine.* Commercial DL-homocysteine was reduced with metallic sodium in liquid ammonia (du Vigneaud, Audrieth & Loring, 1930); excess of ammonia was removed under vacuum and the product dissolved in 0·5 N-HCl and stored at 0° under N_2 ('O_2-free'). It was neutralized with 2 N-NaOH just before use. The stock solution was 0·1 M with respect to DL-homocysteine, but iodometric titration (Lavine, 1935) showed that some homocystine (up to 0·02 M) was also present. To minimize auto-oxidation the homocysteine was always added last to reaction mixtures.

Vitamins. The cobalamin used was Anacobin (British Drug Houses Ltd., London). Members of the folic acid group were as described by Lascelles *et al.* (1954).

Other chemicals. Amino acids and other constituents of the chemically defined media were commercial products recrystallized only if suspected of containing traces of the substances under investigation.

Phosphate buffers were prepared from stock solutions of $Na_2HPO_4,12H_2O$ and KH_2PO_4.

RESULTS

Suspensions of organisms deficient in p-*aminobenzoic acid*

Preparation of suspensions. E. coli 273/384, which requires *p*-aminobenzoic acid for growth on the glucose–ammonium salts medium (*A*), can be grown on a medium devoid of this factor provided that thymine, purines and a mixture of certain amino acids are present (Lampen *et al.* 1949). In the present work these amino acids were supplied either as the pure DL-compounds (supplement *F*) or, with other amino acids, as casein hydrolysate (supplement *E*). In each case μM-*p*-aminobenzoic acid was also added; this is only one-tenth of the concentration required for just visible growth in the basal medium but led to more consistent and somewhat heavier growth with the supplements. It did not affect the requirement for *p*-aminobenzoic acid for methionine synthesis by the harvested organisms.

Requirements for the synthesis of methionine. Such organisms formed methionine when incubated in solution *M*, which contained only buffered glucose, homocysteine and *p*-aminobenzoic acid. There was little or no synthesis in the absence of any of these compounds, though it was just detectable when glucose only was omitted (Table 2). Cobalamin further increased methionine synthesis, but to a variable extent (see also Table 5); it was normally added in all further tests.

The amount of methionine formed was proportional to the concentration of *p*-aminobenzoic acid over the range 0·01–0·1 μM (Fig. 2*a*); 0·01 μM is also about the limiting concentration for growth on the basal medium *A*.

Neither pteroylglutamic acid nor its N^5-formyltetrahydro-derivative (leucovorin) replaces *p*-aminobenzoic acid for the growth of this mutant (Lampen *et al.* 1949; Woods, 1952); they were also inactive (at μM) for the production of methionine by suspensions.

Concurrent synthesis of methionine and folic acid. Although the two synthetic forms of folic acid were inactive, it was possible that other forms were produced by the organism itself from *p*-aminobenzoic

acid and promoted methionine synthesis. Lascelles & Woods (1952; and unpublished work) found suspensions of this strain of *E. coli* to synthesize from *p*-aminobenzoic acid a form of folic acid active for the growth of *Lactobacillus casei* but giving a dose/response curve somewhat different from that for either pteroylglutamic acid or leucovorin.

The conditions necessary for the formation of methionine in solution *M* were also sufficient for the concurrent production of material(s) active for the growth of both *Lactobacillus casei* and *Leuconostoc citrovorum* (Table 3); they were not formed in the absence of *p*-aminobenzoic acid.

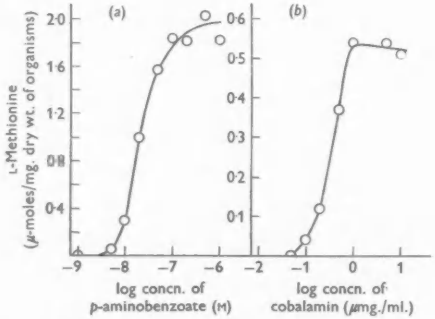

Fig. 2. Effect of the concentration of (*a*) *p*-aminobenzoic acid and (*b*) cobalamin on the synthesis of methionine by suspensions of *E. coli*: (*a*) strain 273/384 and (*b*) strain 200. Organisms (0·33 mg./ml.) were incubated in solution *M* for 16 hr.

Inhibition of synthesis of methionine by sulphathiazole. The utilization of *p*-aminobenzoic acid by strain 273/384 both for growth and for synthesis of folic acid by suspensions is inhibited competitively by sulphonamides (Lascelles & Woods, 1952). The effect of sulphathiazole on methionine production by suspensions of organisms rich and poor in preformed folic acid was tested as follows. Organisms harvested from the medium deficient in *p*-aminobenzoic acid were divided into four portions; one of these (I) was held at 0° while the other parts were further treated. The second portion (II) was incubated with glucose, glutamate and *p*-aminobenzoic acid in order to load the organisms with folic acid (Lascelles & Woods, 1952). The other two portions were similarly incubated except that in one *p*-aminobenzoic acid was omitted (III) and in the other sulphathiazole was added (IV); in both (III) and (IV) the organisms should remain low in folic acid (Lascelles & Woods, 1952). Each batch of treated organisms was re-harvested and washed once on the centrifuge. The effect of *p*-aminobenzoic acid and sulphathiazole on methionine synthesis in solution *M* was then tested (Table 4).

Table 3. *Concurrent formation of methionine and folic acid*

Organisms (strain 273/384) were incubated for 18 hr. in solution *M* plus cobalamin (10 μmg./ml.) with or without *p*-aminobenzoic acid (μM).

Expt. no.	Concn. of organisms (mg./ml.)	*p*-Aminobenzoic acid	Product: L-Methionine (mM)	Product: *Lactobacillus casei* factor (μmM)	Product: *Leuconostoc citrovorum* factor (μmM)
1	0·23	−	0·02	0·1	0·3
		+	0·36	6·4	8·0
2	0·28	−	0·02	<0·1	0·1
		+	0·24	8·0	10·3

Table 4. *Effect of sulphathiazole on synthesis of methionine*

Treatment: *E. coli* 273/384 (0·25 mg./ml.) were incubated for 5 hr. at 37° in 0·067 M-phosphate buffer containing the substances indicated [glucose, 10 mM; L-glutamate, 10 mM; *p*-aminobenzoic acid (*p*-AB), 10 μM; sulphathiazole, mM]. Test for methionine synthesis: Treated organisms (0·25 mg./ml.) were incubated for 13 hr. at 37° in solution *M* plus cobalamin (10 μmg./ml.) with and without sulphathiazole and *p*-aminobenzoic acid as shown.

Additions for test of methionine synthesis: Sulphathiazole (mM)	Additions for test of methionine synthesis: *p*-AB	L-Methionine formed (μmole/mg. of organisms) by organisms previously treated with: Nothing (control) (I)	Glucose, glutamate, *p*-AB (II)	Glucose, glutamate (III)	Glucose, glutamate, *p*-AB, sulphathiazole (IV)
−	−	<0·05	0·06	0·05	<0·05
−	μM	0·74	0·78	0·60	0·56
+	−	<0·05	0·63	0·05	<0·05
+	μM	<0·05	0·68	0·05	<0·05
+	2 mM	0·64	.	.	0·68

Synthesis of methionine by the untreated organisms (I) in the presence of *p*-aminobenzoic acid was inhibited by sulphathiazole and the inhibition was overcome by increasing the concentration of *p*-aminobenzoic acid. The organisms (II) containing preformed folic acid produced, even in the absence of added *p*-aminobenzoic acid, 80 % as much methionine as the untreated batch plus this vitamin. This is not due to carry-over of free *p*-aminobenzoic acid from the treatment system since methionine formation was not in this case inhibited by sulphathiazole. The control batches of treated organisms (III and IV) behaved in essentially the same way as the untreated one; the treatment would not have led to the formation of intracellular folic acid.

These experiments provide support for the idea that the function of *p*-aminobenzoic acid in methionine synthesis is exerted after its conversion into a folic acid-like compound; they do not, however, exclude the possibility that the active substance is not a folic acid but is formed from *p*-aminobenzoic acid by a sulphonamide-sensitive reaction.

Other strains of Escherichia coli. Methionine synthesis by *E. coli* 518 (the parent strain of the mutant used above) and by *E. coli* 200 (a cobalamin auxotroph) was neither dependent on nor stimulated by *p*-aminobenzoic acid; neither strain requires the vitamin for growth and they can presumably synthesize it. A double mutant (Y 44) requiring both *p*-aminobenzoic acid and histidine for growth was again dependent on *p*-aminobenzoic acid for methionine synthesis by harvested suspensions (S. Wijesundera & D. D. Woods, unpublished experiments).

Suspensions of organisms deficient in cobalamin

Preparation of suspensions. Certain mutant strains of *E. coli* (Davis & Mingioli, 1950) will grow on medium *A* only if provided either with cobalamin or methionine. It was likely therefore that growth with methionine would produce organisms at least relatively deficient in vitamin B_{12} compared with other strains. There is so far no satisfactory evidence whether cobalamin auxotrophs can or cannot synthesize the vitamin in quantities insufficient to support growth. *E. coli* 200 harvested from medium *A* containing 0·13 mM-methionine was used for most of the work reported.

Requirements for methionine synthesis. Methionine was formed by organisms incubated in solution *M* only if cobalamin was added (Table 2). The amount synthesized bore a linear relation to the concentration of cobalamin from about 0·1–1·0 μmg./ml. (Fig. 2*b*); 0·1 μmg./ml. supports about 80 % of maximal growth of the organism on medium *A*.

Of the other constituents of solution *M*, homocysteine was essential, but there was 20 % methionine formation (with 'preincubated' organisms only) in the absence of glucose (Table 2).

Effect of anaerobic conditions on the requirement for cobalamin. Dubnoff (1950) has suggested that cobalamin is required for the reduction of homocystine to homocysteine. Control suspensions incubated in solution *M* without cobalamin gave a strongly positive nitroprusside reaction, showing that free thiol groups were still present and that the homocysteine had not become completely oxidized.

Incubation in N_2 or H_2 instead of air did not affect the absolute requirement for cobalamin for methionine production by strain 200. Similarly, it did not increase methionine synthesis by strain PA 15 in the absence of added cobalamin; the synthesis by this organism is increased about five-fold by the vitamin (see below).

Other strains of Escherichia coli. Methionine formation by suspensions of strain 113/3 (another mutant requiring methionine or cobalamin for growth) was also negligible unless cobalamin were added (Table 5).

With five other strains two- to five-fold increases in synthesis were obtained on adding cobalamin to

Table 5. *Effect of cobalamin on synthesis of methionine by various strains of* Escherichia coli

Organisms at the stated concentration were incubated at 37° in solution *M* supplemented as required with μM-*p*-aminobenzoic acid (strain 273/384) or 10 mM-DL-serine (strains PA 15 and 55/5). Cobalamin concentration was 10 μmg./ml., when present.

Strain of *E. coli*	Growth factor required	Concn. of organisms (mg./ml.)	Time of incubation (hr.)	L-Methionine formed (μmoles/mg. of organisms)	
				Without cobalamin	With cobalamin
113/3	Cobalamin or methionine	0·21	16	<0·05	0·67
86	None	0·21	15	0·59	1·80
518	None	0·37*	17	0·10	0·54
273/384	*p*-Aminobenzoic acid	0·25*	13	0·23	0·72
PA 15	Serine	0·32	8	0·57	4·00
55/5	Serine	0·32	8	1·40	2·50

* Preincubated organisms (see Table 2).

solution *M* (Table 5). With the one strain (273/384) tested extensively the increase also varied within these limits with five different batches of organisms. Since the growth of these strains on medium *A* is neither dependent on nor increased by cobalamin it was not expected that it would affect methionine formation by suspensions; it has not been determined whether the organisms are relatively deficient in the vitamin at the time of harvesting or whether losses occur during the preparation of the suspensions.

Serine as source of the methyl group in methionine synthesis

In the experiments so far described no substance was added to the reaction mixture specifically as source of the one carbon atom unit; the carbon atom of the methyl group of methionine must therefore have been derived from glucose, homocysteine or endogenous cell constituents.

Effect of serine and other one-carbon-atom donors. In early experiments with strains requiring *p*-aminobenzoic acid or cobalamin a number of substances (e.g. formate, betaine, choline, glycine), known to act as precursors or sources of methyl groups in animal metabolism, failed to increase methionine synthesis in solution *M*. In nine experiments serine alone gave some increment, though to a highly variable extent (sometimes none at all), with different batches of either organism (Table 6). When active, serine increased both the rate and extent of methionine formation.

Inhibition of methionine synthesis by carbon dioxide. Lascelles *et al.* (1954) found CO_2 plus glycine to replace serine for the growth of *Leuconostoc mesenteroides* P 60, and it was at one time thought possible that CO_2 might serve as ultimate source of a one-carbon-atom unit for the formation of serine from glycine by this organism (Lascelles, Cross & Woods, 1951). Methionine synthesis by suspensions of strain 200 was not increased by an atmosphere enriched with CO_2; on the contrary it was reduced to about at least a half and in some cases was completely suppressed (Table 7). Similar results were obtained with two normal strains (86 and 518), synthesis being reduced by 86 and 41 % respectively. The slight fall in pH in the presence of CO_2 did not account for these results.

Since one possible explanation of the effect of CO_2 was that it prevented the formation of a one-carbon-atom donor, a number of such compounds (and others) were tested for ability to overcome the inhibition of methionine synthesis. DL-Serine (10 mM) restored synthesis almost completely

Table 6. *Effect of serine on synthesis of methionine by* Escherichia coli *strains* 200 *and* 273/384

Organisms were incubated in solution *M* plus (strain 200) 10 μmg. of cobalamin/ml. for 8 hr. or (strain 273/384) μM-*p*-aminobenzoic acid for 8–16 hr.

Strain of *E. coli*	Number of experiments	Concn. of organisms (mg./ml.)	Percentage increase in L-methionine formed (μmoles/mg. of organisms) with serine (10 mM) added
200	6	0·18–0·31	0–55
273/384	3	0·15–0·26	33–154

Table 7. *Inhibition of the synthesis of methionine by carbon dioxide*

E. coli 200 was incubated in solution *M* plus cobalamin (10 μmg./ml.) and other additions, as stated, in open tubes (controls) or in Thunberg tubes evacuated and filled with 20 % (v/v) CO_2 in air.

	Expt. no.				
	1	2	3	4	5
Concn. of organisms (mg./ml.)	0·28	0·28	0·32	0·24	0·21
Time of incubation (hr.)	16	16	16	9	8
L-Methionine formed in control (μmoles/mg. of organisms)	1·29	2·40	0·44	0·95	0·65
Addition (10 mM)	Percentage suppression of methionine formation by CO_2				
None	84	76	100	49	41
DL-Serine	.	3	13	1	3
Choline	83	.	.	49	.
Formate	83	.	.	51	.
Betaine	.	.	.	47	.
Glycine	82	.	.	50	43
DL-Threonine	.	.	.	.	40
DL-Homoserine	.	.	.	.	38

(Table 7), but no other substance, including a number of amino acids not listed, had any detectable effect. DL-Serine still had pronounced activity at 1·0 mM, but none at 0·1 mM.

Strains requiring either serine or glycine for growth. The ability of serine both to increase methionine synthesis (albeit to a variable extent) and to overcome the inhibition of such synthesis by CO_2 suggested that it might be the main source of the one-carbon-atom unit for condensation with homocysteine. The strains of *E. coli* so far used did not require added serine for growth and were presumably able to synthesize it from constituents of the culture medium. Since a source of both carbon (glucose) and nitrogen (homocysteine) was present, serine may also have been formed by suspensions in solution *M*. Together with the known ability of *E. coli* to catabolize serine (Chargaff & Sprinson, 1943), this may well account for the erratic results noted above.

The best way of determining the absolute effect of serine on methionine formation appeared to be the use of mutants requiring serine for growth and presumably unable to synthesize it. Two strains (PA 15 and 55/5) were available which required either serine or glycine for growth on medium *A* at 37°; synthesis of serine by suspensions was therefore likely to be limited unless glycine was present. Strain 55/5 grew at 25° without these amino acids and at this temperature must be considered potentially able to synthesize them.

Suspensions of strain PA 15 formed no detectable methionine in solution *M*, even with cobalamin added, unless serine was present (Table 8). Similar results were obtained with strain 55/5 at 37°; at 25°, however, although good methionine synthesis was not obtained unless serine was present, there was a small but reproducible synthesis in the absence of added serine (Table 8). This result is in accord with the growth requirements of the strain, which suggest that it can make some serine at 25°.

The following amino acids and possible one-carbon-atom donors (each tested at both 10 and 1 mM) failed to replace serine for methionine synthesis by either strain PA 15 or 55/5 or both: each of the amino acids listed in the *Leuconostoc mesenteroides* basal medium (except threonine), L-hydroxyproline, DL-α-aminobutyric acid, formate, choline, betaine, xanthine, ethanol and glycollic acid. Glycine and, to a lesser extent, DL-threonine and DL-homoserine had activity under certain conditions to be described later.

The results with both strains strongly support the view that serine is the main one-carbon-atom donor for methionine formation by *E. coli* and that strains able to achieve synthesis of serine in growing cultures can also do so to some extent under the conditions of incubation in solution *M*.

Requirement for vitamins in the presence of serine. If serine is a normal reactant in methionine formation it is possible that the requirements for *p*-aminobenzoic acid and cobalamin demonstrated with strains 273/384 and 200 were for the synthesis of serine rather than its condensation with homocysteine. There is evidence (reviewed by Woods, 1952) that members of the folic acid group are

Table 8. *Effect of serine on synthesis of methionine by serine/glycine auxotrophs*

Organisms harvested from medium *A* with (PA15) or without (55/5) 10 mM-serine were incubated (0·31 mg./ml.) in solution *M* in the presence or absence of serine (mM).

Strain of *E. coli*	Incubation: Temp.	Incubation: Time (hr.)	L-Methionine formed (μmoles/mg. of organisms): Serine absent	L-Methionine formed (μmoles/mg. of organisms): Serine present
PA15	37°	8	<0·05	1·85
55/5	37	16	<0·05	3·70
	25	16	0·13	3·00

Table 9. *Dependence of synthesis of methionine on* p*-aminobenzoic acid and cobalamin when serine is present*

Vitamin-deficient organisms (0·21 mg./ml.) were incubated for 17 hr. in solution *M* with the additions shown.

Strain of *E. coli*	Additions: Cobalamin (10 μmg./ml.)	Additions: *p*-Aminobenzoic acid (μM)	Additions: Serine (10 mM)	L-Methionine formed (μmoles/mg. of organisms)
273/384 (deficient in *p*-aminobenzoic acid)	+*	+	+	2·00
	+	−	+	<0·05
	+	+	−	0·89
	+	−	−	<0·05
200 (deficient in vitamin B_{12})	+	−	+	0·89
	−	−	+	<0·05
	+	−	−	0·65
	−	−	−	<0·05

* Cobalamin was added in all cases with strain 273/384 since it is known (Table 5) markedly to increase methionine formation.

Table 10. *Concurrent formation of methionine and glycine*

Organisms at the stated concentration were incubated for 8 hr. at 37° in solution *M* supplemented with cobalamin (10 μmg./ml.) and, where indicated, with serine (10 mM).

E. coli strain	Growth medium	Concn. of organisms (mg./ml.)	Addition to solution *M*	Product (μmoles/mg. of organisms) L-Methionine	Glycine
PA 15	*A* + serine	0·34	.	<0·05	0·15
			Serine	1·40	1·21
PA 15	*A* + glycine	0·27	.	<0·05	0·15
			Serine	2·85	0·96
55/5	*A**	0·24	.	<0·05	<0·10
			Serine	2·34	1·74

* Growth temperature 25°.

required for the formation of serine from glycine by several bacteria, and it was at one time thought that cobalamin had a function in serine–glycine interconversion in animal tissues (see discussion by Arnstein, 1955).

There was still an absolute requirement for *p*-aminobenzoic acid and cobalamin for synthesis of methionine by suspensions of strains 273/384 and 200 respectively, even though preformed serine was present (Table 9); it is clear therefore that both factors are required for the condensation reaction apart from any function they may or may not have in serine formation.

Production of glycine. There is good evidence that glycine is the other main product of the reaction between homocysteine and serine. With strain 200 (a cobalamin auxotroph) methionine formation is not dependent on added serine, which it can presumably synthesize. Methionine and glycine were produced in approximately equimolar quantities whether or not serine was added, but no glycine was formed from serine when cobalamin was omitted and methionine synthesis could not occur (Table 12). Either the formation of glycine is linked to the methylation of homocysteine or the formation of glycine from serine itself requires cobalamin.

With strains (PA 15, 55/5) which cannot synthesize serine at the temperature of the test, methionine formation required added serine and, in a number of experiments, 0·75–0·9 mole of glycine was found for each mole of methionine (Table 10). With strain 55/5 a ratio near unity was maintained almost throughout the period of methionine production (Fig. 3); with strain PA 15 glycine formation decreased in rate more sharply and at an earlier stage than did that of methionine.

A strict molecular equivalence of methionine and glycine would not be expected, and would be of doubtful significance for two reasons. First, the organisms cause slight, though unequal, apparent destruction of both amino acids in conditions comparable with the synthesis tests. Thus suspensions of strain PA 15 removed (per mg. of organism in 4 hr.) 0·3 and 0·55 μmole of methionine and glycine

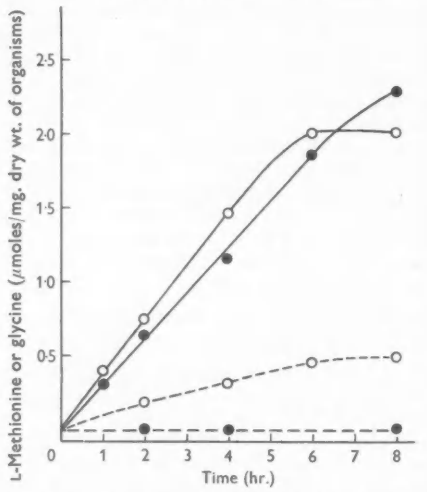

Fig. 3. Formation of methionine and glycine by suspensions of *E. coli* 55/5. Organisms (0·23 mg./ml.) were incubated in solution *M* plus DL-serine (10 mM) and cobalamin (10 μmg./ml.). ●, Methionine; ○, glycine; —, homocysteine present; - - -, homocysteine omitted from solution *M*.

respectively. Secondly, there is some production of glycine from serine in the absence of homocysteine (Fig. 3); it is not known to what extent, if any, this is suppressed when methionine synthesis can occur.

When strain PA 15 was grown originally on glycine instead of serine, the yield of glycine was only one-third of that of methionine (Table 10); possible reasons for this have not been investigated.

Further experiments with mutants requiring serine or glycine for growth

Growth on serine or glycine in relation to methionine synthesis by suspensions. E. coli PA 15 will grow on medium *A* supplemented with either serine or glycine and can presumably therefore

form either amino acid from the other. Organisms harvested from a serine-containing medium utilized this amino acid, presumably as a one-carbon-atom donor, for methionine formation from homocysteine; glycine, however, showed only just detectable activity in this respect (Fig. 4). When the organisms were grown with glycine the activity of this amino acid was greatly increased, and after incubation for 8 hr. the suspensions had formed about 75% as much methionine as with serine as donor (Fig. 4). The results suggest than an adaptive process, involving the presence of glycine itself during growth, is required to produce the enzyme systems concerned in the activation of glycine as one-carbon-atom donor. This is supported by the fact that even with glycine-grown cells there is an increasing rate of methionine synthesis with time (Fig. 4).

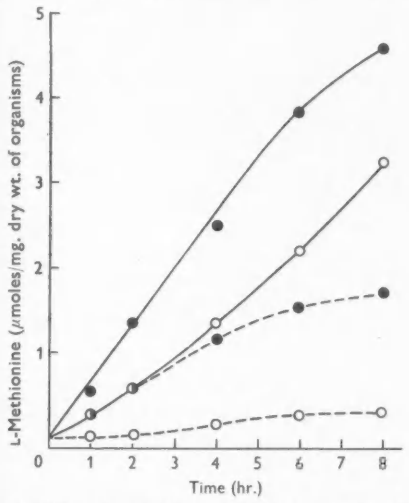

Fig. 4. Effect of growth in serine or glycine on the utilization of these amino acids as one-carbon-atom donors by *E. coli* PA 15. Organisms (0·3 mg./ml.) were incubated in solution *M* plus cobalamin (10 μmg./ml.) and with either DL-serine (10 mM) or glycine (10 mM). ●, Serine; ○, glycine as one-carbon-atom donor. Organisms were grown on serine (- - -) or glycine (—).

Surprisingly, growth on glycine in place of serine also more than doubled both the rate of production and final yields of methionine when serine was used as one-carbon-atom source (Fig. 4); this result was consistently obtained and will be discussed later.

Threonine and homoserine as one-carbon-atom donors; conversion into glycine. Suspensions of strain PA 15 cannot synthesize serine or glycine from constituents of solution *M*; the ability of related compounds to replace these amino acids as source of the single-carbon-atom unit may thus be conveniently tested with this strain.

A large number of amino acids and other compounds were tested, but only threonine and homoserine were effective (Table 11) and then only with organisms harvested from growth on glycine; serine-grown organisms were inactive. Since growth on glycine is essential for the utilization of glycine itself, and since neither threonine nor homoserine was as effective as glycine, it was possible that the two hydroxyamino acids were first converted into glycine before acting as one-carbon-atom donors. This was tested directly by assaying glycine as well as methionine (Table 11); glycine was found to be produced from both threonine and homoserine whether or not the conditions (presence or absence of homocysteine) permitted methionine synthesis. Formation of glycine from the hydroxyamino acids was further confirmed with suspensions of strain 200 (a cobalamin auxotroph); here methionine synthesis can be controlled by the presence or absence of cobalamin.

Table 11. *Formation of methionine and glycine from various one-carbon-atom donors by a serine/glycine auxotroph*

Suspensions of *E. coli* PA 15 harvested from growth on medium *A* plus either serine or glycine were incubated for 8 hr. at 37° in solution *M* plus cobalamin (10 μmg./ml.), with homocysteine omitted as shown. Source of single-carbon-atom unit (10 mM-DL-) was added as indicated. Concn. of organisms: serine-grown, 0·34 mg./ml.; glycine-grown, 0·27 mg./ml.

Grown with	Homocysteine in solution *M*	Product formed (μmoles/mg. of organisms)				
		No addition	With serine	With glycine	With threonine	With homoserine
				Methionine formed		
Serine	Absent	<0·05	<0·05	<0·05	<0·05	<0·05
Serine	Present	<0·05	1·40	0·11	<0·05	<0·05
Glycine	Present	<0·05	2·85	1·95	0·84	0·37
				Glycine formed		
Serine	Absent	0·15	0·43	—	0·82	0·88
Serine	Present	0·15	1·21	—	0·59	0·59
Glycine	Present	0·15	0·96	—	—	—

Table 12. *Methionine and glycine formation from various one-carbon-atom donors by a cobalamin auxotroph*

E. coli 200 (0·26 mg./ml.) was incubated in solution *M* for 8 hr. at 30° with cobalamin (10 μmg./ml.) added as shown. Amino acid supplements (10 mM-DL-) were added as indicated.

	Product formed (μmoles/mg. of organisms)				
Cobalamin	No addition	With serine	With glycine	With threonine	With homoserine
	Methionine formed				
Absent	<0·05	<0·05	<0·05	<0·05	<0·05
Present	0·60	0·69	0·53	0·73	0·67
	Glycine formed				
Absent	0·20	<0·10	—	1·72	0·96
Present	0·40	0·67	—	1·04	0·82

In the absence of methionine formation glycine was formed from homoserine and threonine, but not from serine (Table 12). When methionine was synthesized there was a slight decrease in the amount of glycine formed from threonine and homoserine. With this strain (as opposed to PA 15) it was noted that the utilization of glycine was not dependent on previous growth on glycine since the growth medium did not contain this amino acid.

It is concluded that the apparent activity of homoserine and threonine as sources of the one-carbon-atom unit may be accounted for by their prior conversion into glycine.

Methionine synthesis by a normal strain

The results described in preceding sections were obtained with a variety of mutant strains of *E. coli*, not all derived from the same parent. It was thought advisable to attempt to obtain similar evidence with a single prototrophic strain, even though the effects could not be expected to be absolute because of the ability of the prototroph to synthesize the metabolites in question. Strain 518 (the parent strain of the *p*-aminobenzoic acid auxotroph 273/384) was chosen for this work; the complete system used for synthesis was solution *M* plus serine (10 mM) and cobalamin (10 μmg./ml.).

Serine as one-carbon-atom donor. The presence of serine increased methionine synthesis by 40%; furthermore, an atmosphere of 20% (v/v) of CO_2 decreased synthesis by about 40%, the inhibition being overcome completely by serine.

Effect of cobalamin. The inclusion of cobalamin increased methionine formation by a factor of 2·5–5·0, both in the presence and absence of serine (see also Table 5). As with all other strains not requiring cobalamin for growth, the effect of this vitamin was clear-cut but variable.

Folic acid. *p*-Aminobenzoic acid had no effect on methionine synthesis by this strain, which is known to synthesize both *p*-aminobenzoic acid and folic acid (Lascelles & Woods, 1952); the organisms have presumably a full complement of folic acid at the time of harvesting. Attempts were made to obtain suspensions relatively deficient in folic acid by growing the cultures in sub-inhibitory concentrations of sulphathiazole (0·5 μM) followed by incubation of the harvested organisms in buffered glucose for 6 hr. This reduces the folic acid content of the organisms (Lascelles & Woods, 1952) but not apparently to a sufficient extent, since there was still no reduction of methionine synthesis, compared with normal controls, and no effect of *p*-aminobenzoic acid was found.

Confirmatory tests for methionine and glycine

The identification of methionine and glycine as products of the reaction between homocysteine and serine rests on the specificity of the assay organism (*Leuconostoc mesenteroides* P 60). Although this was entirely satisfactory within the limits of the checks made, certain ancillary tests were carried out. For these experiments the two serine/glycine mutants (PA 15 and 55/5) were used since they produced the largest quantities of methionine and glycine. For controls serine was omitted from the synthesis system.

Glycine. Organisms were removed by centrifuging from the heated products of synthesis experiments and the supernatant was chromatographed on columns of Dowex 50 resin (see the Methods section). A peak corresponding to glycine was obtained. The concurrent formation of alanine made a satisfactory quantitative estimation of glycine impossible with the quantities formed. There was no positive correlation between the formation of alanine and methionine; it was shown that at least one source of alanine was a transamination reaction between homocysteine and pyruvate, the latter being presumably derived from serine or glucose.

Methionine. Supernatants from synthesis experiments were chromatographed on paper with butanol–acetic acid and phenol–water as solvents. A ninhydrin-positive spot with the same R_F as

synthetic methionine was detected by microbiological assay. Acid-hydrolysis of the samples before chromatography did not affect the result, but peroxide treatment destroyed this ninhydrin-positive material. Similarly, peroxide destroyed the activity of the samples for growth of *L. mesenteroides* P 60.

In a further experiment with the products formed by strain PA 15, two samples, one from the control without serine and the other from a complete synthesis system, were chromatographed in duplicate for 18 hr. with butanol–acetic acid; a solution of methionine was similarly treated. The papers were dried at room temperature and one of each set of duplicates was sprayed with ninhydrin to locate the spots. The other strips were cut into 2 cm. squares and each section was folded and placed in methionine assay medium (2 ml.). After autoclaving and inoculation with *L. mesenteroides* P 60 the tubes were incubated as for a methionine assay. Growth occurred only in those tubes corresponding to the spot given by synthetic methionine and the spot with the same R_F obtained from the complete synthesis system.

Samples from experiments with strain 200 (cobalamin auxotroph) and strain 273/384 (*p*-aminobenzoic acid auxotroph) had to be added in relatively large amounts to detect a spot corresponding to methionine. The experiment was satisfactory with phenol–water as solvent but with butanol–acetic acid ninhydrin-positive material with R_F only slightly greater than that of methionine was detected in controls from which the vitamins had been omitted and in which no methionine was detectable by microbiological assay. The nature of this substance was not further investigated in the present work.

DISCUSSION

It is not proposed to discuss the present results extensively since they have formed the basis of further experiments at the cell-free enzyme level on the role of folic acid and cobalamin; preliminary reports of some of these studies have been published (Cross & Woods, 1954; Szulmajster & Woods, 1955; Helleiner & Woods, 1956; Kisliuk & Woods, 1957; Helleiner, Kisliuk & Woods, 1957).

It is likely that the serine → glycine reaction is the ultimate main source of single-carbon-atom units for the methylation of homocysteine by suspensions of *E. coli*. Not only is serine an obligatory requirement for methionine synthesis by strains which cannot synthesize serine, but glycine always accompanies methionine as a product with those strains (e.g. the prototrophs) which do not require serine as a reactant. The only other substances found to act (and less efficiently) as one-carbon-atom donors were glycine and amino acids metabolically convertible into it (homoserine, threonine). Formaldehyde, a likely possible donor, could not be expected to act as it combines spontaneously with homocysteine to yield 1:3-thiazane-4-carboxylic acid (Wriston & Mackenzie, 1957), which is metabolically inert in the present system (unpublished experiments of I. Maclean in this Laboratory).

The greatly increased efficacy of serine as donor when strain PA 15 is harvested from growth on glycine rather than serine is puzzling and has not so far been explained. The parallel observation with the same strain that growth on glycine is essential for the activity of glycine as donor could be explained by some type of adaptive enzyme formation. The nature of the other product formed from glycine when it acts as donor has not been investigated. Unpublished experiments with [^{14}C]glycine by M. J. Cross in this Laboratory showed that serine was not formed as an intermediate. Kizer, Speck & Aurand (1955*a*, *b*) have obtained indirect evidence that serine (perhaps in the form of phosphoserine) is required for methionine synthesis from homocysteine by growing cultures of *Streptococcus lactis*.

The experiments on the parallel inhibition by sulphonamide of the synthesis of both methionine and of folic acid derivatives gives strong, though indirect, evidence that *p*-aminobenzoic acid is converted into some form of folic acid before it exerts its function, presumably as a one-carbon-atom carrier, in the synthesis of methionine. In extension of this work Szulmajster & Woods (1955) have briefly reported that a dialysed cell-free preparation from acetone-dried organisms required a cofactor whose properties were akin to those of a natural coenzyme form of folic acid (Wright, 1955). Tetrahydropteroylglutamic acid cannot replace this factor (Cross & Woods, 1954) but can do so if the organisms are originally grown on a medium containing added cobalamin (Kisliuk & Woods, 1957). A preparation made by sonic disintegration can also use tetrahydrofolic acid, but only if cobalamin is also added (Helleiner *et al.* 1957). In recent work with sheep-liver homogenates Nakao & Greenberg (1958) give evidence that tetrahydropteroylglutamic acid functions as a cofactor in the overall methylation of homocysteine by serine or formaldehyde; cobalamin, however, had no effect.

Cobalamin was required absolutely for methionine synthesis by suspensions of mutants requiring this factor (or methionine) for growth. It has since been briefly reported (Helleiner & Woods, 1956) that sonic disintegrates show the same requirement. Suspensions of all strains (whether cobalamin-requiring or not) showed marked, though variable, increases in methionine synthesis when cobalamin was added. A possible explanation is

that the washed suspensions are partially deficient in cobalamin, owing either to inherent lack of synthetic ability or to losses during the preparation of suspensions. Alternatively, it is possible that the normal mechanism of methionine synthesis does not require cobalamin, but that this vitamin permits an alternative and more efficient mechanism of conversion of homocysteine into methionine.

SUMMARY

1. Washed suspensions of a number of normal and mutant strains of *Escherichia coli* converted homocysteine into methionine; the latter was determined by microbiological assay with *Leuconostoc mesenteroides* P 60.

2. By the use of auxotrophic mutants unable to synthesize *p*-aminobenzoic acid and cobalamin respectively, it was shown that both these vitamins are essential for the reaction.

3. Synthetic forms of folic acid did not replace *p*-aminobenzoic acid. Sulphathiazole, however, inhibited both the synthesis of methionine and the conversion of *p*-aminobenzoic acid into folic acid.

4. Serine is the probable ultimate source of the methyl group. Methionine is not formed by mutants requiring serine or glycine for growth unless serine (or, under limited conditions, glycine) is added.

5. Glycine is formed concurrently with methionine whether or not it is essential to add serine.

6. Glycine, threonine and homoserine also act as one-carbon-atom donors, though less efficiently than serine. There is evidence that threonine and homoserine must first be converted into glycine.

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The Pentosanases of some Rumen Bacteria

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A study of the hydrolysis of some soluble pentosans by suspensions of sheep-rumen bacteria was previously reported (Howard, 1957). It was shown that the mixture of bacteria normally present in the rumen is able, by removal of arabinose side chains if present, and by apparently random breaking of the xylan chain, to hydrolyse the pentosans completely into their constituent monosaccharides. The next step in this investigation was to examine pure cultures of those organisms of the rumen flora which are active in pentosan decomposition. From a number of such cultures which have been isolated in this Laboratory, two were selected for detailed study. One was *Bacteroides amylogenes* (Doetsch, Howard, Mann & Oxford, 1957), isolated from sheep rumen on a medium containing xylose. The other, which bears the number 11 in our Laboratory collection, was a strain isolated from bovine-rumen contents on a medium containing wheat-flour pentosan, and tentatively assigned to the genus *Butyrivibrio* (Bryant & Small, 1956). A brief description of this organism is given in the Appendix to this paper.

Preliminary experiments showed that intact cells from pentosan cultures of each of these bacteria vigorously hydrolysed the pentosan, and attention was then turned to the preparation of active cell-free materials. The properties of such preparations are the subject of this paper. Attempts have been made to separate the component enzymes of the pentosan-hydrolysing complex, and to determine their properties and some of the factors controlling the production of them by the bacteria.

MATERIALS AND METHODS

Bacterial cultures. The strain of *Bacteroides amylogenes* used was that described by Doetsch *et al.* (1957). The *Butyrivibrio* sp. was isolated in 1957 from the rumen of a heifer fed on silage. Both cultures used in this work were obtained from freeze-dried samples prepared soon after the original isolations. Stock cultures were maintained on the medium described below, solidified with 2% of agar and containing xylose instead of the wheat-flour pentosan. Subcultures were made once a month and stored in the refrigerator. Cultures were incubated at 38°.

The bacteria from two 2-day slope cultures on pentosan medium, suspended in sterile 0·9% NaCl, were used as inoculum for each 250 ml. of liquid culture.

Preparation of media. The following stock solutions were used in preparing the culture media: I, 0·3% K_2HPO_4. II, A solution containing 0·3% of KH_2PO_4, 0·6% of $(NH_4)_2SO_4$, 0·6% of NaCl, 0·06% of $MgSO_4,7H_2O$ and 0·06% of $CaCl_2$. III, A solution of 0·625% of cysteine hydrochloride and 6% of $NaHCO_3$. IV, 2·5% (w/v) wheat-flour pentosan. Solution III was sterilized by passage through a Seitz filter and solution IV by steaming for 30 min. on 3 successive days.

Bottles of 300 ml. capacity, containing 250 ml. of liquid medium, were used for large-scale culture. Into each bottle were placed solutions I and II (37·5 ml. each), Difco yeast extract (1·25 g.), strained rumen liquor (25 ml.), 0·1% solution of resazurin sodium (L. Light and Co. Ltd.; 0·25 ml.) and water (79 ml.). The mixture was sterilized by heating the bottles in the autoclave for 15 min. at 15 lb./in.^{2} pressure and, after cooling, was saturated with sterile CO_2. Finally, sterile solution III (21 ml.) and sterile solution IV (50 ml.) were added with sterile precautions. The technique of Hungate (1950) was used throughout this work, i.e. the cultures were always grown in rubber-stoppered tubes or bottles under an atmosphere of sterile, O_2-free CO_2, and a flow of this gas into the tube or bottle was maintained whenever one was opened.

Carbohydrate materials. The following substances were used. (1) The soluble pentosan (araboxylan) from wheat flour. The ratio xylose:arabinose in the wheat-flour pentosan was about 1·77:1. (2) Rhodymenan, the soluble xylan of the red seaweed *Rhodymenia palmata*. The preparation of these was described by Howard (1957). (3) Xylan (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). This material as purchased contained an appreciable amount of arabinose and a little glucose and galactose. It was used without further purification in culture media, but for use as a substrate in enzyme experiments it was purified once through the copper complex as described by Sørensen (1957). The ratio xylose:arabinose in the product was 12:1. (4) The series of β-1:4-linked xylose oligosaccharides, from xylobiose to xylohexaose, separated from a partial hydrolysate of wheat-flour pentosan (Howard, 1957). Xylobiose was crystalline; the other four materials were chromatographically homogeneous amorphous solids or syrups. (5) The mixture of arabinose- and xylose-containing oligosaccharides obtained from the same hydrolysate as mentioned in (4) above, and referred to as fraction 12 in Table 1 of Howard (1957). This material, which was eluted during chromatography on charcoal by 30% (v/v) ethanol, had a mean degree of polymerization (DP) of 11·6, and contained no oligosaccharides of DP less than 6. Complete acid hydrolysis yielded approximately equal amounts of xylose and arabinose. Hydrolysis with N-acetic acid at 100° for 4 hr. (Professor I. A. Preece, personal communication) yielded much arabinose and traces of xylobiose and xylotriose as the only chromatographically identifiable substances. This material will be

referred to in this paper as 'mixed oligosaccharides' to distinguish it from the other oligosaccharides composed only of anhydroxylose units. (6) D-Glucose, L-arabinose and D-xylose were AnalaR or Kerfoot's Biochemical Reagents. For use in culture media all carbohydrates were sterilized separately in 2·5% (w/v) solution or suspension in water, polysaccharides by intermittent steaming (see above), simple sugars by filtration through Berkefeld candles and xylobiose by filtration through a small sintered-glass filter, grade 5/3.

Preparation of freeze-dried powders from bacterial cultures. (*a*) Large-scale preparation from cells only. After an appropriate period of incubation, usually about 5 days, the bottles containing the cultures were transferred to the refrigerator and left overnight. Residual pentose and pH were measured, and a Gram-stained smear was examined microscopically to check the purity of the culture. The cells were sedimented by centrifuging at 0° at 22 000 *g* for 30 min. The sediment, after draining as free as possible of culture fluid, was suspended in water with the aid of a glass homogenizer. Sufficient butanol to make two layers was added with shaking, the mixture stored at 0° overnight and then centrifuged at 0° at 22 000 *g* for 30 min. The clear aqueous layer was removed, the butanol and bacterial debris were dispersed in a little water and a second aqueous extract was obtained by centrifuging. The combined extracts, having a volume of about one-tenth that of the original culture, were filtered through a sintered-glass filter, grade 5/3, and dialysed, first against running tap water for 36 hr. and finally overnight at 1° against distilled water. The contents of the dialysis sacs were distributed as layers less than 1 cm. deep in glass beakers, and freeze-dried. Disks of filter paper, fastened over the beakers with rubber bands, prevented loss of the powder on opening the machine. The product was a very fluffy, pale buff-coloured powder, readily soluble in water to a pale-brown solution. One litre of culture yielded about 50 mg. of powder.

(*b*) Small-scale preparation from whole cultures. When the bacteria were grown on diffusible carbohydrates it was possible to obtain preparations containing both the enzymes excreted into the medium and those associated with the cells. To the culture, grown in a small screw-capped bottle, was added enough butanol to make two layers. The bottle was well shaken and stored in the refrigerator overnight. The subsequent procedure was the same as described above, and yielded between 20 and 30 mg. of freeze-dried powder from 15 ml. of culture.

Preparation and storage of enzyme solutions. The freeze-dried powder was incubated with the appropriate buffer solution for 30 min. and the trace of insoluble matter removed by centrifuging. The clear supernatant was removed and, if not required for use immediately, was stored in the refrigerator under toluene. Except in the electrophoretic experiments, McIlvaine's (1921) phosphate–citrate buffers were used throughout this work.

Measurement of enzyme activity. (i) Xylanase. Wheat-flour pentosan or xylan was used in this assay. The former substrate (200 mg.) was dissolved in water (100 ml.) to yield a slightly opalescent solution which was stable for a few weeks in the refrigerator. A solution of the xylan was prepared by the method of Sørensen (1957). Unlike the xylan used by Sørensen, that used in this work formed a copious precipitate when the alkaline solution was dialysed. A turbid solution containing about half the starting material was obtained after centrifuging. The proportions of arabinose, glucose and galactose in an acid-hydrolysed portion of this solution were no greater than in the purified xylan. After determination of the total pentose concentration in the xylan solution, it was diluted with water to a concentration of 2 mg./ml. (as xylose) and stored in the refrigerator. Both polysaccharide solutions were filtered through Whatman no. 1 paper before use. Enzyme assay was carried out in tubes marked at 25 ml. Each tube contained polysaccharide solution (0·5 ml., i.e. about 1 mg. of substrate), together with enzyme and buffer solution to a total vol. of 1 ml. The tubes, and appropriate controls, were placed in a water bath at 38° for 4 hr. A portion (1 ml.) of the alkaline copper reagent of Nelson (1944) was added and reducing sugar estimated colorimetrically. The reducing sugar was expressed as xylose after correction for the small reducing power of the polysaccharides when incubated in the absence of enzyme, and the result used to calculate the apparent percentage hydrolysis of the xylan or pentosan. It was found unnecessary to deproteinize the solutions before carrying out the reducing-sugar determination.

(ii) Xylobiase. The procedure was similar to that in (i) above. Each tube contained xylobiose (0·5 ml. of aqueous 0·01% solution, i.e. 50 μg.), and incubation was for 1 hr. only. The reducing value of the solutions was corrected for that of xylobiose alone, determined in a control tube without enzyme.

(iii) 'Cellulase' and cellobiase. Our colleague, Dr G. N. Festenstein, kindly tested some of our materials for hydrolytic activity against sodium carboxymethylcellulose and cellobiose. Hydrolysis of the former substrate was measured by a reducing-sugar method, hydrolysis of the latter by a specific test for glucose employing glucose oxidase (Festenstein, 1958*a*, *b*).

Qualitative test for enzyme activity. Approximately equal volumes of substrate solution (2%, w/v) and buffered enzyme solution were incubated under toluene in sealed capillary tubes (Porter & Hoban, 1954) for between 18 and 48 hr. The solution was then transferred to paper which had been treated with $HgCl_2$ (Bealing & Bacon, 1953), and chromatograms were prepared with the techniques and solvent systems described previously (Howard, 1957). It was occasionally necessary to examine the products of hydrolysis of polysaccharides when the hydrolysis had proceeded to only a small extent. In these cases the solution to be tested was treated with 2 vol. of ethanol, and the precipitated polysaccharide removed by centrifuging. The supernatant solution was then deionized and concentrated for paper chromatography.

Thermal-stability experiments. A tube containing buffer solution (10 ml., pH 6·5) was deeply immersed in a water bath maintained at 60°. After allowing a period for equilibration of temperature, a solution of the enzyme in the same buffer solution (2 ml.) was quickly added and mixed, and a sample (2 ml.) was immediately transferred to a tube chilled in ice. Further samples were withdrawn while the enzyme solution was kept at 60°. The chilled samples of heated enzyme were stored under toluene in the refrigerator until tested for activity.

Electrophoretic methods. Zone electrophoresis was in columns of starch; the apparatus used has been described by Mould & Thomas (1958) and Mould & Stephen (1958). 0·05 M-Sodium phosphate buffer, pH 6·5 or 7·5, was used,

Table 1. *Enzyme activity of preparations from two strains of rumen bacteria after growth on various carbohydrates*

(*a*) *Bacteroides amylogenes*; (*b*) *Butyrivibrio* sp. +, Enzyme activity present; 0, enzyme activity absent; −, not tested.

	Substrates for enzyme tests											
					Wheat-flour pentosan*				'Mixed oligosaccharides'*			
	Xylobiose		Xylan		(*a*)		(*b*)		(*a*)		(*b*)	
Growth substrate	(*a*)	(*b*)	(*a*)	(*b*)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Glucose, arabinose, xylose	0	0	0	0	0	0	0	0	0	0	−	−
Xylobiose	+	−	0	−	0	+	−	−	+	+	−	−
Wheat-flour pentosan	+	+	+	+	+	+	+	+	+	+	+	+
Rhodymenan	+	−	+	−	+	+	−	−	+	+	−	−
Xylan	−	+	−	+	−	−	+	+	−	−	+	+

* The two symbols for each entry in these columns refer to (1) the breaking of xylose–xylose linkages, and (2) the breaking of arabinose–xylose linkages.

and a p.d. of 500 v was applied to the electrodes. In some experiments the column was cooled by a flow of water at 1° through the jacket, in others no temperature control was applied and the column was at, or a little above, room temperature (about 17°).

Other methods. Pentoses were determined by a modification of Drury's (1948) method, and protein by the method of Lowry, Rosebrough, Farr & Randall (1951). Chromatography on charcoal was as described by Howard (1957).

RESULTS

Effect of growth medium on production of enzymes. The two strains of bacteria were grown on media containing different substances as sole source of fermentable carbohydrate. When the carbohydrate was a polysaccharide the final preparation contained the enzymes associated only with the bacterial cells [method (*a*) above]; when growth was on a diffusible carbohydrate, the final preparation contained also any enzymes secreted into solution [method (*b*) above]. The effects of these products on xylobiose, wheat-flour pentosan, xylan and 'mixed oligosaccharides' were determined. Hydrolytic action was assessed quantitatively by measuring the increase in reducing power, and qualitatively by paper chromatography. The results are summarized in Tables 1 and 2. The preparations which decomposed xylobiose had a solely hydrolytic action when the concentration of the substrate was 1 or 2%. Enzyme preparations from cultures grown on the polysaccharides hydrolysed the wheat-flour pentosan and the xylan in all cases tested. Xylose and small amounts of xylose–oligosaccharides were present in the hydrolysates; the wheat-flour pentosan and 'mixed oligosaccharides' hydrolysates also contained some arabinose. In experiments with the latter substrate rupture of xylose–xylose linkages was detected by the formation of xylose or chromatographically detectable oligosaccharides or both.

Table 2. *Activity of enzyme preparations from two strains of rumen bacteria*

Activity is expressed as μg. of xylose released from 1 mg. of substrate in 1 hr. by 1 mg. of enzyme powder, when incubated under the conditions described in the text. (*a*) *Bacteroides amylogenes*; (*b*) *Butyrivibrio* sp.

	Substrates for tests			
	Xylan		Wheat-flour pentosan	
Growth substrate	(*a*)	(*b*)	(*a*)	(*b*)
Glucose, arabinose, xylose	<1	<1	<1	<1
Xylobiose	<1	—	10	—
Wheat-flour pentosan	30	—	220	160

The clear supernatant solutions obtained by centrifuging the cultures when grown on the polysaccharides, and which contained the unused substrate, were usually discarded. A little enzyme activity was present in these solutions, such that traces of xylose, arabinose and oligosaccharides could be produced on incubation with wheat-flour pentosan. The low activity, and the presence of large amounts of unused polysaccharide substrate, rendered unprofitable any further study of the enzymes secreted into solution by the bacteria.

Electrophoretic experiments. Enzyme preparations from cultures of both bacterial strains grown on wheat-flour pentosan, rhodymenan and xylan were examined. No significant differences were found in the behaviour of the enzymes in the preparations. Whether the pH of the solution was 6·5 or 7·5 and whether the temperature was 1° or 17° did not affect the movement of the enzymes. Whatever the source of crude enzyme, and whatever the conditions, all the enzyme activity moved towards the anode. When the ability of the eluate fractions to hydrolyse xylobiose or wheat-flour pentosan was examined it was seen that at least two different enzymes were present, and that they

were partially separated by electrophoresis (Fig. 1). Some enzymically inert protein moved ahead of the more mobile enzyme, and the greatest enzyme activity per unit weight of protein was found in the least mobile fractions. Portions of the eluates were incubated with wheat-flour pentosan and xylobiose, and the resulting sugars examined chromatographically. The more mobile fractions attacked both substrates, and produced from the pentosan substantial amounts of xylose and arabinose, as well as smaller quantities of oligosaccharides. The less-mobile enzyme fractions yielded less monosaccharide but the proportion of arabinose to xylose became higher. The least-mobile fractions produced no monosaccharides but yielded traces of xylobiose and other oligosaccharides. Appropriate fractions from the electrophoretic separation were pooled to give two solutions, one containing most of the xylobiase activity with a trace of the xylanase, the other containing most of the xylanase, free from xylobiase. A portion of the xylanase solution was freed from buffer salts by dialysis and freeze-dried. A white powder resulted which was much less readily soluble in water than the original crude enzyme. When subjected to electrophoresis under the same conditions as before only a single peak of xylanase activity was observed. Some tailing of enzyme occurred.

Properties of xylobiase

Sources. Three sources of this enzyme were available: the products of electrophoresis of crude enzyme from cultures of *Bacteroides amylogenes* and the *Butyrivibrio* sp. on wheat-flour pentosan, and the crude enzyme from the culture of *Bacteroides amylogenes* on xylobiose. These will be referred to as *A*, *B* and *C* respectively. *A* and *B* were slightly contaminated with xylanase, *C* was not. In some experiments the presence of the xylanase was immaterial, but in others it was necessary to use completely xylanase-free material. In such experiments only xylobiase *C* was used. No difference in properties between the xylobiases of the two bacterial strains was observed.

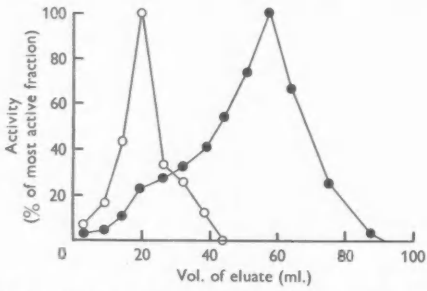

Fig. 1. Zone electrophoresis of *Butyrivibrio* sp. enzymes in 0·05 M-sodium phosphate buffer, pH 7·5, for 19 hr. Temp., 1°; applied p.d., 500 v. ○, Xylobiase activity; ●, xylanase activity. For assay conditions see text.

Action pattern. Chromatographic tests showed this enzyme to be active not only against xylobiose but against all the other xylose–oligosaccharides, at least up to xylohexaose. When the concentration of xylobiose was 1 or 2% (w/v) the action of the enzyme appeared to be purely hydrolytic, no sign of transferring activity being apparent at any stage of the reaction. Xylobiose was progressively, and at length completely, converted into xylose. Xylotriose yielded xylobiose and xylose, initially in roughly equal proportions, but later, when all but a trace of substrate had been decomposed, most of the xylobiose had also been hydrolysed. The first products of action on xylotetraose were xylotriose and xylose; xylobiose did not appear until later in the reaction and was always a minor constituent of the mixture. When nearly all the xylotetraose had disappeared, the mixture contained traces of xylotriose and xylobiose, and much xylose. The first products of action on xylopentaose were xylotetraose and xylose; xylotriose and xylobiose appeared successively after this, and in the early stages of hydrolysis the concentration of the intermediates always decreased in the order xylotetraose, xylotriose and xylobiose. At the end of the reaction the substances disappeared from the mixture in the order xylopentaose, xylotetraose, xylotriose and xylobiose. A similar train of events was observed during hydrolysis of xylohexaose.

A series of fractions from the electrophoresis of crude enzyme from *Bacteroides amylogenes* was tested for β-xylosidase activity (Conchie, 1954). (We are indebted to our colleague Dr J. Conchie for this determination.) The ability of the different fractions to hydrolyse phenyl β-xyloside closely parallelled their ability to hydrolyse xylobiose.

A portion of xylobiase *C* was incubated with solutions of the oligosaccharides (xylobiose to xylohexaose) in each of which the concentration was 100 μg./ml. as xylose, pH 6·6. Reducing sugar was determined in 1 ml. samples taken at the beginning of the experiment and after incubation for 3 hr. The activity of the enzyme, as measured by the increase in reducing power of each test substance, was found to decrease slightly as the oligosaccharide series was ascended (Fig. 2).

Neither the crude enzyme preparation nor the electrophoretically prepared xylobiase (xylobiase *B*) from a culture of the *Butyrivibrio* sp. on wheat-flour pentosan was able to hydrolyse cellobiose. There was at least a 1000-fold difference between the cellobiase and xylobiase activities.

The xylobiase preparations *A* and *B* produced on long incubation with wheat-flour pentosan a

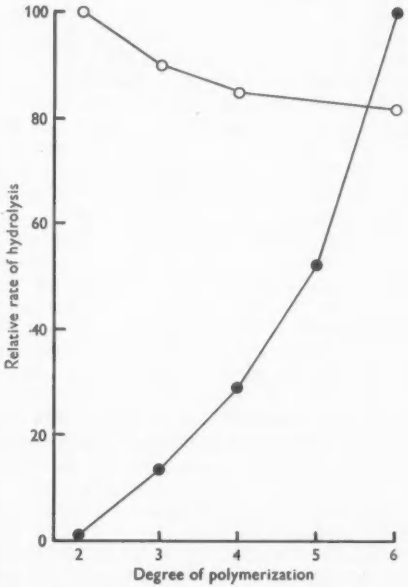

Fig. 2. Rates of hydrolysis of xylose oligosaccharides by enzymes of *Bacteroides amylogenes*. ○, Xylobiase; ●, xylanase. For conditions see text.

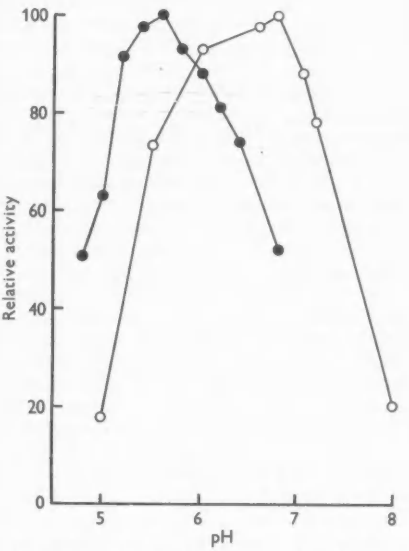

Fig. 3. Variation of activity of enzymes of *Butyrivibrio* sp. with pH. ○, Xylobiase; ●, xylanase. For conditions see text.

little xylose and a trace of arabinose. Xylobiase *C*, on the other hand, had a much slighter effect on the pentosan, arabinose being the only recognizable product, and had no effect at all on xylan.

pH *optimum and thermal stability*. Xylobiase *B* had an optimum pH of 6·8, when tested in phosphate–citrate buffer during 1·5 hr. against xylobiose (Fig. 3). The activity of xylobiase *C* was destroyed within 5 min. when heated at 60° in buffer, pH 6·5. This was demonstrated by reducing-sugar determinations after incubating the heated solutions with xylobiose for 4 hr. and confirmed chromatographically after incubating for 40 hr.

Arabinosidase activity. In addition to removing arabinose from the pentosan, the various xylobiase preparations also removed this sugar from the 'mixed oligosaccharides'. In each case xylose was the only other chromatographically identifiable substance formed. The electrophoretically prepared xylobiase fraction yielded more xylose, and xylobiase *C* yielded more arabinose, under identical conditions. The portion of the last-named material which had been heated at 60° for 5 min. produced arabinose but no xylose from the 'mixed oligosaccharides' and portions which had been heated for 10 min. or longer produced neither xylose nor arabinose.

Transferring activity. Xylobiose (22·6 mg.) and freeze-dried enzyme preparation from the *Butyrivibrio* sp. (2·3 mg.) were dissolved in buffer solution, pH 6·5, diluted 1:10 with water (0·1 ml.). Small drops were transferred to a $HgCl_2$-treated chromatogram paper at intervals during incubation for 24 hr. Development of the chromatogram showed that all but a trace of the xylobiose had disappeared during the experimental period, and that the concentration of xylose in the mixture increased throughout. In addition, small quantities of two other substances appeared transitorily on the chromatogram. The major substance was evident after incubation for 1 hr., the minor substance after 2 hr., but both had disappeared after

Table 3. *Charcoal-column separation of the products of action of xylobiase on xylobiose*

Xylobiose (0·25 g.) was dissolved in 1·2 ml. of diluted buffer solution, pH 6·5.

Fraction no.	Ethanol in eluate (%, v/v)	Wt. of fraction (mg.)	Provisional identification
1	0	—	Xylose
2	5	82	Xylobiose
3	7·5	3	Rhodymenabiose*
4	10	15	Xylotriose
5	12·5	13	3^2-β-Xylosylxylobiose*
6	15	—	Tetrasaccharide (?)*

* Howard (1957).

24 hr. The two substances were chromatographically identical with xylotriose and 3^2-β-xylosylxylobiose (Howard, 1957) respectively.

In a large-scale experiment, xylobiose (0·25 g.) and xylobiase *C* (8·5 mg.) were dissolved in the diluted buffer solution (1·2 ml.) and incubated for 17 hr. The resulting mixture of sugars was separated by elution with aqueous ethanol from a charcoal column and the fractions shown in Table 3 were obtained. The provisional identification of the substances is based on their chromatographic mobilities when run on the same paper as authentic material, and also on the order of their elution from the column.

Properties of xylanase

Action pattern. Enzymes attacking the xylan chain, in either xylan or wheat-flour pentosan, were less mobile than the xylobiase in the electrophoretic conditions used. No difference in behaviour between the xylanases of the two bacterial strains was observed. The more mobile of the xylanase fractions, which were contaminated with a little xylobiase (Fig. 1), yielded some xylose when acting upon xylan, and some xylose and arabinose from the pentosan. The less-mobile fractions, however, produced no monosaccharides from the pentosan and only traces of xylose from the xylan. When the enzyme in these fractions acted upon the xylan there was an initial rapid increase in reducing power, followed by a longer period during which hydrolysis was much slower. Chromatographically identifiable substances were produced only during the second stage of hydrolysis. Xylobiose was the main end product, and smaller amounts of other oligosaccharides were also formed. No chromatographically identifiable substances were formed from the pentosan; in a quantitative test, the reducing power of the enzyme–pentosan mixture became constant at a value suggesting that the pentosan had been broken down into oligosaccharides with DP of about 12.

The xylanase, like the xylobiase, acted upon the xylose oligosaccharides but in a completely different manner. A quantitative test, carried out under the same conditions as used for the xylobiase, showed that the activity of the xylanase decreased sharply as the DP was reduced (Fig. 2). The hydrolysis of xylobiose was extremely feeble, and could be detected chromatographically only after prolonged incubation. A slightly greater effect on xylotriose could be observed, small, roughly equal amounts of xylobiose and xylose being formed. Xylotetraose was much more readily hydrolysed; much xylobiose, and traces of xylotriose and xylose, appeared. The main products from xylopentaose were xylotriose and xylobiose, with a trace of xylose but no xylotetraose. Xylohexaose yielded considerable amounts of xylotriose, with some xylobiose and xylotetraose and a trace of xylose, but no xylopentaose.

An unfractionated enzyme preparation from the *Butyrivibrio* sp. did not hydrolyse carboxymethylcellulose. The xylanase activity of the preparation was at least 100 times its 'cellulase' activity.

Arabinosidase activity. The electrophoretically more mobile xylanase fractions were able to remove arabinose from the wheat-flour pentosan and from the 'mixed oligosaccharides'; the less mobile fractions could not do so. The earlier of the fractions active in this respect hydrolysed xylobiose, but the later ones did not.

pH *optimum and thermal stability.* During incubation for 4 hr. in phosphate–citrate buffer solution, the activity against xylan had an optimum pH of 5·6 (Fig. 3). When heated at 60° at pH 6·5 about two-thirds of the activity against xylan was destroyed in 15 min., but some remained even after heating for 60 min.

DISCUSSION

The two strains of bacteria used in this work have many bacteriological similarities, and the similarity extends to their pentosan-decomposing enzymes. Although it has not been possible to compare the enzymes of the two strains in every particular, all the results which have been obtained suggest that these enzyme systems are identical. For the purposes of this discussion, no distinction will be drawn between results obtained by the use of enzymes from the two sources.

The behaviour of the enzyme preparations, both unfractionated and partially purified, towards the various substrates leads to the conclusion that the decomposition of pentosans by the two bacterial strains involves three enzymes: a xylanase, a β-xylosidase and an arabinosidase. The xylanase is the enzyme responsible for the initial breakdown of the xylan chain. It is an adaptive or inducible enzyme, produced only when the bacteria are growing in a medium containing xylan or a related polysaccharide. Under these conditions it is not the only pentosan-decomposing enzyme formed, and experiments with crude preparations do not indicate with certainty the properties of the enzyme itself. The small scale on which this work had to be done prevented us from preparing pure protein fractions by the conventional techniques. We have had some moderate success, however, in separating the different enzyme activities by zone electrophoresis, and we believe that the least-mobile fractions, although not necessarily containing only one protein, do contain only one of the enzymes involved in pentosan decomposition, namely the xylanase.

The mode of action of this enzyme shows it to be a typical endoenzyme, in that it splits more readily the xylosidic linkages near the middle of the chain. In so far as the xylanase attacks oligosaccharides, the same preference for inner linkages is found. Thus xylotetraose was split almost wholly at its central linkage to yield xylobiose. Xylopentaose was split mainly at one or other or both of the two inner linkages; attack at either of the two outer linkages was negligible, since no xylotetraose was detected during hydrolysis. Xylohexaose was similarly not attacked at the outer linkages; since the yield of xylotriose was considerably greater than the yield of xylotetraose plus xylobiose, the central linkage is presumably more accessible to the enzyme than are the two adjacent ones. The presence of arabinose units as side chains attached to the xylan appeared to inhibit the action of the xylanase. In the wheat-flour pentosan used in this work there was rather more than one arabinose to every two xylose units, i.e. if the side chains were uniformly distributed there would be a substituent on alternate xylose units. Xylose–xylose units protected in this way are apparently not accessible to the xylanase, and the observed partial decomposition of the pentosan is only possible because of uneven distribution of arabinose along the xylan chain (Perlin, 1951). The xylan used in these experiments, on the other hand, contained only one arabinose for every 12 xylose units, and so was readily attacked. Bishop & Whitaker (1955) observed that a purified cellulase from *Myrothecium verrucaria* hydrolysed the xylose–xylose linkages, but not the arabinose–xylose linkages, in a specimen of wheat-straw xylan. The converse effect, of a xylanase hydrolysing cellulose or its derivatives, does not seem to occur; the bacterial xylanase caused no detectable hydrolysis of carboxymethylcellulose.

The enzyme referred to above as 'xylobiase' is also inducible, but it is produced not only when xylobiose is the substrate, but also during growth on the polysaccharides. By cultivation of the bacteria on a medium containing xylobiose, the adaptive natures of the xylanase and xylobiase permitted the preparation of the latter freed from the former enzyme. This method yielded a purer specimen of xylobiase than could be obtained by electrophoresis. The enzyme is active against a wide range of xylose-containing oligosaccharides, upon each of which it acts by stepwise removal of single xylose units. The close parallel between the activities of the electrophoretic fractions against xylobiose and phenyl β-xyloside also suggests that this enzyme is a fairly non-specific β-xylosidase. It appears from Fig. 2 that the enzyme could attack oligosaccharides higher than the hexaose, and it is in fact able to release xylose from the 'mixed oligosaccharides' but is without effect on the xylan.

The mode of action of the two enzymes separately suggests that the joint action of the xylanase and xylobiase on xylan is to cause a gradual fragmentation of the chain, leading finally to xylose as sole end product. This is exactly the pattern of action of the suspensions of mixed rumen bacteria (Howard, 1957) and of the unfractionated enzyme preparations from the two bacterial strains. Among the various oligosaccharides present during xylan hydrolysis, it is clear that no firm distinction can be made between those which are substrates of the xylanase only, and those which are substrates of the xylobiase only. There is considerable overlapping of the activities of the two enzymes; as the xylan chains become shorter they become less susceptible to the action of the xylanase and more susceptible to the action of the xylobiase.

Although purely hydrolytic in its action on dilute solutions of xylobiose, the xylobiase demonstrated a slight transferase activity when acting upon a concentrated solution. Other hydrolytic enzymes showing transferase activity under similar conditions, e.g. invertase (Allen & Bacon, 1956) and cellobiase (Crook & Stone, 1957), attach the majority of transferred glycosyl residues to primary alcohol groups in the receptor molecule. In the xylose–oligosaccharides there are, of course, no such groups, and probably for this reason the yield of synthetic oligosaccharides from xylobiose was very low. Crook & Stone (1957) observed that in transferring glucosyl residues to the secondary alcohol groups of cellobiose, a fungal cellobiase attached most to C-4 of the pyranose ring, less to C-3 and very little to C-2. A similar preference for that part of the molecule farthest from the glycosidic linkage was found in the bacterial xylobiase, leading to xylotriose as major product and 3^2-β-xylosylxylobiose as minor product of transferase action on xylobiose.

The primary problem at present about the ability of the enzyme preparations to remove arabinose units from wheat-flour pentosan or the 'mixed oligosaccharides' is whether this is the work of a separate arabinosidase or is another aspect of the action of the xylanase or xylobiase. Conclusive proof of the existence of a separate arabinosidase would be afforded by the preparation of an active fraction free from xylanase and xylobiase activity. We have not yet encountered such material. We have, however, found arabinosidase activity in a xylobiase preparation free from xylanase, and also in some electrophoretically prepared xylanase solutions which were free from xylobiase. Furthermore, the arabinosidase activity of the xylobiase preparation seemed slightly more resistant to heat than did the xylobiase activity.

For these reasons we consider the arabinosidase to be a separate entity, not identical with either the xylanase or the xylobiase. This enzyme, like the other two, is inducible but in a curious way. It is not produced during growth on monosaccharides, but appears whenever either of the other two enzymes is formed. Hence it is produced during growth on xylobiose or rhodymenan, neither of which contains arabinose. Nothing further can be said at present about the properties of this enzyme, except that its electrophoretic mobility is intermediate between those of xylanase and xylobiase.

SUMMARY

1. Two strains of xylan-fermenting bacteria, one from the sheep and the other from the bovine rumen, have been grown in media containing a wide variety of xylans and related carbohydrates. Cell-free extracts have been prepared and tested for hydrolytic activity.

2. Two enzymes have been separated from the whole extracts by zone electrophoresis in starch columns.

3. The less mobile enzyme, referred to as xylanase, is formed by the two bacteria when these are grown on xylan or similar polysaccharide. It acts upon xylan by hydrolysing inner linkages, producing xylobiose as the main end product. It hydrolyses oligosaccharides similarly but has negligible action on xylotriose and xylobiose. Its effect on the xylan is inhibited by the presence of arabinose side chains.

4. The electrophoretically more mobile enzyme, referred to as xylobiase, is produced by the two bacteria when these are grown on xylose polysaccharides or xylobiose. It is a non-specific β-xylosidase, acting upon oligosaccharides by removal of single xylose units, and also hydrolyses phenyl β-xyloside. When acting upon a 20% (w/v) solution of xylobiose, this enzyme synthesizes small amounts of two trisaccharides, xylotriose and 3^2-β-xylosylxylobiose.

5. Some evidence is presented for the existence in the crude enzyme preparations of a discrete arabinosidase, which removes the arabinose side chains present in soluble pentosans. It appears to be formed by the bacteria whenever either xylanase or xylobiase is produced.

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APPENDIX

Isolation and Properties of the Xylan-Fermenting Bacterium 11

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The isolation and properties of the bacterium 11 (Institute collection) used in the preceding study of the degradation of xylans are described here.

METHODS

The medium used for isolation was the same as that described in the above paper for maintenance of stock cultures, but without yeast extract and containing wheat-flour pentosan (0·25%). It was later found that better growth occurred with yeast added. Since growth occurred on this medium without added carbohydrate the medium used for determining the substrates utilized was altered in that the rumen fluid was replaced by Bacto-tryptone (0·2%) and Bacto-peptone (0·2%). In the presence of a suitable substrate this medium gave growth about equal to that of the rumen fluid medium. Very little growth

occurred on this medium in the absence of utilizable carbohydrate. Substrates were added as sterile solutions (sterilized by filtration, except for polysaccharides which were sterilized by steaming) to give a final concentration of 0·5 or 1%. Utilization of substrate was tested by comparing the growth on slopes of the appropriate medium with growth on the basal medium, after one subculture on the substrate-containing medium. A maximum incubation time of 8 days was used to see if growth occurred. Colonies were tested with iodine solution after 24 and 48 hr. to determine whether formation of intracellular starch-like polysaccharide occurred during growth of the cells. Tests for motility and other properties were made by the methods described by Doetsch, Howard, Mann & Oxford (1957). For determination of the products of fermentation of xylose the rumen fluid in the liquid medium described in the paper above was replaced by water, leaving the yeast extract (0·5%). The cells were centrifuged off before analysis of the supernatant fluid. Total volatile fatty acids were determined by titration of a steam-distillate, after acidification. Qualitative tests for the presence of different volatile fatty acids were made by paper chromatography (Elsden & Lewis, 1953; Burness & King, 1958). Quantitative determinations were made by chromatography on silicic acid columns (Keeney, 1955). Formic acid was determined colorimetrically (Grant, 1947), and by difference after distilling the initial volatile acid distillate with mercuric salts (Friedemann, 1938). Lactic acid was determined on the original culture supernatant by the method of Barker & Summerson (1941). Succinic acid was not determined.

Isolation of the organism. Rumen fluid, taken via a permanent rumen cannula from a heifer 4 hr. after feeding on grass silage, was diluted tenfold by the use of the anaerobic technique and diluting fluid described by Doetsch, Robinson & Shaw (1952); glucose was omitted from the diluting fluid. A portion (0·5 ml.) of diluted sample was transferred to 5 ml. of liquid rumen fluid–pentosan–agar medium in test tubes and the medium allowed to solidify with the stoppered tubes laid flat. Colonies from the primary medium were subcultured on to slopes of the same medium. The growth on these slopes was usually mixed and so the dilution procedure was repeated until a pure culture was obtained.

RESULTS

Streptococcus bovis grew along with curved Gram-negative rod-like bacteria in up to 10^6–10^8 dilutions of the rumen fluid. From these curved rods three isolates were selected for further study, one being that numbered 11. The properties of this bacterium differed somewhat from those of the other two, but only this one will be described here.

Properties of the organism. The bacterium was non-motile under the conditions of test and flagella were not observed. The bacterium was a strict anaerobe, no growth taking place on media in which the resazurin was oxidized. Cells were Gram-negative, usually curved and $0{\cdot}3\,\mu \times 1{\cdot}8\,\mu$, occurring singly or sometimes in short chains. The cells were somewhat pleomorphic and in older cultures filamentous forms (up to $6{\cdot}5\,\mu$) and bulbous forms appeared. No intracellular starch-like polysaccharide was detected. Colonies on pentosan–agar medium were whitish, round, smooth and opaque. Hydrogen sulphide was formed, but catalase and indole tests were negative. Growth occurred at 29° and 38°, but not at 55°.

Substrates on which growth occurred are shown in Table 1. Since the media contained carbonate no evaluation of metabolic gas production was made. The proportions of the products of fermentation of xylose varied somewhat with rate of growth etc. in different experiments, but formic acid, acetic acid, propionic acid and butyric acid were formed, in one experiment in the proportions 35, 13·9, 26·9 and 17·2% of the total volatile acids formed. Lactic acid was also produced, in one experiment in a concentration of 0·144 m-equiv./5 ml. of culture, compared with a total volatile-acid concentration of 0·160 m-equiv./5 ml. of culture. These concentrations accounted for 69·5% of the carbon of the xylose originally present in the medium.

Table 1. *Fermentation reactions of rumen organism* 11

The medium is described in the text. All substrates were at 1% concentration except those marked with an asterisk, which were at 0·5%. +, − and ±: Growth, no growth and little growth respectively, in the presence of substrate.

Substrate	Reaction	Substrate	Reaction	Substrate	Reaction	Substrate	Reaction
D-Xylose	+	Glycerol	−	Cellobiose	+	Amylose*	−
L-Arabinose	+	Ribitol*	−	Trehalose	+	Starch	−
D-Ribose	−	D-Mannitol	±	Melibiose	+	Cellulose	−
L-Rhamnose	+	D-Sorbitol	+	Raffinose	+	D-Glucurono-lactone*	−
L-Fucose*	+	Dulcitol	+	Methyl α-D-glucoside	+	Pyruvate	+
D-Glucose	+	*meso*-Inositol	−	Salicin	+	DL-Lactate	−
D-Mannose	+	D-Glucosamine–HCl	−	Aesculin	+	Fumarate	−
D-Galactose	+	Sucrose	+	Inulin	−	Succinate*	−
D-Fructose	+	Maltose	+	Dextrin	+	Citrate	−
L-Sorbose	−	Lactose	+	Glycogen	−	D-Glucose 1-phosphate	−

DISCUSSION

Bacterium 11 differs from *Bacteroides amylogens* (Doetsch *et al.* 1957) in some of its fermentation reactions, and the fact that it does not form an iodophilic polysaccharide excludes it from classification with this organism. Bryant and his coworkers have described a number of Gram-negative rod-like bacteria isolated from bovine-rumen contents, and bacterium 11 could be classified as a strain of *Butyrivibrio fibrisolvens* (Bryant & Small, 1956*a*). The fact that it rapidly ferments xylan precludes it from inclusion with the species *Succinivibrio dextrinsolvens* (Bryant & Small, 1956*b*) and *Bacteroides succinogenes* (Hungate, 1950), and suggests its inclusion with *Butyrivibrio fibrisolvens*. Although the type species of *B. fibrisolvens* was motile, it was suggested that the 'less actively cellulolytic rod' of Hungate was a *Butyrivibrio* species and this was non-motile (Bryant & Small, 1956*a*). The fermentation reactions of *B. fibrisolvens* strains are variable, but are generally similar to those of bacterium 11, and all are non-iodophilic.

Bacterium 11 differs from *Bacteroides amylogenes* in the products of fermentation of xylose, especially in the formation of lactic acid. However, a lactic acid fermentation of xylose is known, for instance with *Lactobacillus pentosus* (Gest & Lampen, 1952). Although lactic acid is not a final product of the fermentation of xylose by mixed rumen organisms (Doetsch, Robinson, Brown & Shaw, 1953), the work of Pazur, Shuey & Georgi (1958) suggests that it might be an intermediate product.

SUMMARY

The isolation and properties of a xylan-fermenting bacterium from the rumen are described. It was tentatively assigned to the genus *Butyrivibrio*.

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The Structure of a Disaccharide Liberated by Lysozyme from the Cell Walls of *Micrococcus lysodeikticus*

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Salton (1956) studied in some detail the components produced by the action of egg-white lysozyme on the isolated cell walls of certain Gram-positive micro-organisms. In addition to several large fragments which would not diffuse through cellophan, he found that a considerable proportion of the digest was diffusible. He stated that the main 'small fragment' present in the diffusible fraction was possibly a disaccharide of *N*-acetylglucosamine and the unknown amino sugar present in many cell walls (subsequently characterized, e.g. Strange & Kent, 1959, as muramic acid), and adduced certain evidence to support this idea. The work described here confirms the presence of such an oligosaccharide in lysozyme digests of cell wall. The supposition that it is a disaccharide is found to be correct and its structure is established.

EXPERIMENTAL

Methods

Isolation of walls and preparation of lysozyme digests. *Micrococcus lysodeikticus* (N.C.T.C. 2665) was grown in surface culture and the cell walls were isolated as previously described (Perkins & Rogers, 1959). For preparation of digests the isolated walls (100 mg.) were suspended in 0·1 M-ammonium acetate buffer, pH 6·1 (25 ml.), and incubated at 37° with lysozyme (crystalline; The Armour

Laboratories) at a final enzyme concentration of 50 μg./ml. for 3 hr. The essentially clear digest was freeze-dried. The residue was dissolved in a small volume of water and dialysed with agitation in cellophan tubes (Visking, 65 mm. diam.) against three changes of distilled water (10 vol.), each time overnight at 2°. The combined diffusible portions and the non-diffusible portion were each freeze-dried. With the diffusible portion the drying process was repeated several times at 0·001 mm. Hg pressure, in order, as far as possible, to remove ammonium acetate.

Paper chromatography and zone electrophoresis. Chromatograms were developed either in butan-1-ol–acetic acid–water (63:10:27, by vol.; organic phase) or in butan-1-ol–pyridine–water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951). Carbohydrate material was detected either by the procedure for hexosamines described by Partridge (1948), the final heating after spraying with *p*-dimethylaminobenzaldehyde reagent being omitted, or by aniline phthalate (Partridge, 1949). For preparative purposes Whatman no. 3 chromatography paper was washed before use by the method of Hanes, Hird & Isherwood (1952) to remove peptide contaminants. Zone electrophoresis was carried out on Whatman no. 3 paper either in acetic acid (2 N) or collidine–acetate buffer, pH 6·5 (Newton & Abraham, 1954), at 15 v/cm. in an apparatus similar to that described by Kunkel (1954).

Oxidation with alkaline iodine solution. A portion (50 μl.) of a solution containing not more than 1 μmole of material to be examined, an excess (0·4 ml.) of iodine solution (0·01 N) and 50 μl. of carbonate–bicarbonate buffer (equal volumes of 0·2 M-Na_2CO_3 solution and 0·2 M-$NaHCO_3$ solution, pH 9·9) were mixed together and kept in a glass-stoppered tube at 2° overnight. Then with the tube still kept cool, excess of iodine was removed by passing a stream of CO_2 through the mixture. After acidification with HCl, any further iodine formed by oxidation of liberated HI was again removed by a stream of CO_2, and suitable samples were then hydrolysed in N-HCl in a sealed ampoule at 105° for 2 hr. The HCl was removed in a vacuum desiccator over NaOH flakes, and the material was again evaporated to dryness with two further volumes of water before being used for zone electrophoresis.

Formation of chromogens. *N*-Acetylhexosamines, on heating in alkaline solution, yield chromogens which on addition of Ehrlich's reagent give a purple colour (Morgan & Elson, 1934). In the experiment described below where it was desired to separate the chromogens by electrophoretic or chromatographic procedures, the presence of excess of salt was avoided by using triethylamine or triethylamine–carbonate buffer to provide an alkaline medium which was volatile *in vacuo*. The chromogens produced gave the same absorption curve after the addition of Ehrlich's reagent as those obtained by the use of sodium carbonate or borate. The sample was placed in an ampoule together with either 0·05 ml. of triethylamine (redistilled and stored over NaOH) or 0·1 ml. of triethylamine–carbonate buffer [1·5 ml. of triethylamine and 10 ml. of water, dissolved by addition of CO_2; a 20% (v/v) solution in water gave pH 9·5] and water was added to a final volume of 0·5 ml. The sealed ampoule was heated in a boiling-water bath for the chosen period. After cooling, the water and buffer were removed in a vacuum desiccator over NaOH and H_2SO_4. Chromogens were identified on paper by spraying with Ehrlich's reagent alone (Partridge, 1948) without previous heating of the paper. Under these conditions *N*-acetylhexosamines themselves give no reaction.

Estimation of hexosamines. After hydrolysis of samples in HCl (4 N) for 4 hr. at 105° in sealed ampoules, glucosamine and muramic acid were separated and estimated as described by Perkins & Rogers (1959).

Estimation of apparent N-acetylhexosamine. The method of Reissig, Strominger & Leloir (1955) was employed, but for the oligosaccharide the time of heating was increased until maximum colour yield had been obtained (30 min.) (see Perkins, 1960).

Materials

Muramic acid. This was obtained by isolation on a charcoal–Celite column from a hydrolysate of purified cell walls of *Micrococcus lysodeikticus*.

N-Acetylmuramic acid. This was obtained from a partial acid hydrolysate of the cell walls (Perkins & Rogers, 1959) and was isolated from a preparative chromatogram run on washed paper. The material ran on chromatograms in a variety of solvents in the same position as muramic acid which had been *N*-acetylated, gave colour in the *N*-acetylhexosamine test and on acid hydrolysis yielded muramic acid as the only product identifiable on chromatograms by ninhydrin or aniline phthalate.

Glucosaminic acid. This was a crystalline specimen prepared according to the method of Wolfrom & Cron (1952) and Pringsheim & Ruschmann (1915).

N-Acetylglucosamine and glucosamine hydrochloride. These were commercial specimens.

RESULTS

Isolation of oligosaccharide. A lysozyme digest of cell walls was prepared as described above and separated into diffusible and non-diffusible fractions. The diffusible portion was dissolved in water and placed as a streak on washed paper. The chromatogram was developed with butanol–pyridine–water for 20 hr. at 22°. Thin strips sprayed with aniline phthalate showed material which had not moved far from the origin and a well-defined band which had moved about 14 cm. ($R_{N\text{-acetylglucosamine}}$ 0·85). Both these bands gave a purple colour with the hexosamine spray. The unsprayed region of the paper containing the fast-running component was eluted with water and the eluate dried in a vacuum desiccator. This material was found to be an oligosaccharide corresponding to the 'small fragment' described by Salton (1956).

Composition of the oligosaccharide. The isolated oligosaccharide ran as a single component on chromatograms developed with butanol–acetic acid–water and butanol–pyridine–water ($R_{N\text{-acetylglucosamine}}$ 0·85 in each system) and gave a single anionic band on zone electrophoresis at pH 6·5. On acid hydrolysis (2 N-HCl; 2 hr. at 105°) it yielded glucosamine and muramic acid, no amino acids and no glucose. Samples of the isolated material were hydrolysed in HCl and their content of glucosamine and muramic acid was estimated.

On unhydrolysed samples the amount of apparent *N*-acetylglucosamine was determined by comparing the maximal absorption at 585 mμ obtainable by prolonged heating with the maximal absorption given by known amounts of *N*-acetylglucosamine. As stated by Salton (1956), the absorption curve given by the oligosaccharide under these conditions is essentially the same as that given by *N*-acetylglucosamine. The results given in Table 1 indicate that the oligosaccharide contains equimolecular proportions of glucosamine and muramic acid. From the fact that lysozyme digests of cell walls do not contain appreciable amounts of hexosamine with free amino groups (Ingram & Salton, 1957; Perkins, 1960) it is concluded that both amino sugars in the oligosaccharide are *N*-acetylated.

Free reducing group of the oligosaccharide. Since the compound gave a reaction with aniline phthalate and with *N*-acetylhexosamine reagents, at least one of its component hexosamines must have had a free reducing group. The number of units of the two amino sugars in each molecule was not known, although the fast movement on paper chromatograms suggested that it was not likely to be larger than a disaccharide. An attempt was made to answer these problems by oxidation in the cold with alkaline iodine solution (Kuhn, Gauhe & Baer, 1954*a*). Under these circumstances the sugar with a terminal reducing group should be oxidized to the corresponding substituted hexonic acid, leaving the rest of the molecule untouched. Examination of the compounds present in an acid hydrolysate of the oxidized product should therefore indicate the number of units present in the original oligosaccharide and which amino sugar possessed a free reducing group.

Samples of the isolated oligosaccharide were oxidized with cold alkaline iodine solution and the product was hydrolysed in HCl. The hydrolysate was examined by zone electrophoresis in acetic acid. This system gave a clear separation of glucosamine, glucosaminic acid, muramic acid and its oxidized product. It was found that *N*-acetylmuramic acid treated as described above did not yield a product which could be identified with ninhydrin, although iodine was used up and free muramic acid (after acid hydrolysis) disappeared. The procedure of Rydon & Smith (1952), however, which detects amide linkages by treatment with Cl_2 followed by a starch–iodide spray, showed a clear characteristic band after electrophoresis of the product obtained from *N*-acetylmuramic acid by oxidation and acid hydrolysis. It seemed possible that this product might have been due to spontaneous lactam formation, but this point has not been investigated further. The results of these procedures are set out in Table 2. Since no glucosaminic acid at all was formed from the oligosaccharide it can be concluded that C-1 of each molecule of *N*-acetylglucosamine was bound in a glycosidic linkage. Since the oxidized product of muramic acid was clearly present, the free reducing group of the oligosaccharide must have been that of *N*-acetylmuramic acid. Furthermore, as at the same time all the free muramic acid had disappeared, the material must have been a disaccharide rather than a tetrasaccharide or higher polymer. This is in accord with the relatively rapid movement of the material on paper chromatograms.

Table 1. *Composition of the disaccharide material and amount of 'apparent* N*-acetylglucosamine'*

Sample no.	Before acid hydrolysis: Apparent *N*-acetylglucosamine* (mole)	After acid hydrolysis: Glucosamine (mole)	After acid hydrolysis: Muramic acid (mole)
1	0·37	1·0	0·85
2	0·39	1·0	0·90

* 'Apparent *N*-acetylglucosamine' is the maximal absorption at 585 mμ obtainable by prolonged heating in the Reissig, Strominger & Leloir (1955) conditions compared with the maximal absorption given by *N*-acetylglucosamine.

Table 2. *Zone electrophoresis of products of alkaline iodine oxidation followed by acid hydrolysis*

Electrophoresis was performed on paper at 15 v/cm. for 250 min. in 2 N-acetic acid.

Material	Treatment	Method of detection	Distance moved towards cathode (cm.)
Glucosamine hydrochloride	None	Ninhydrin	24·2
Glucosaminic acid	None	Ninhydrin	10·0
Muramic acid	None	Ninhydrin	14·8
N-Acetylglucosamine	Alkaline I_2 + acid hydrolysis	Ninhydrin Cl_2 + starch–KI	10·0 10·0 (none at 2·8)
N-Acetylmuramic acid	Alkaline I_2 + acid hydrolysis	Cl_2 + starch–KI	2·8
Oligosaccharide	Alkaline I_2 + acid hydrolysis	Cl_2 + starch–KI Ninhydrin	2·8 24·2 (none at 10·0)

Linkage between C-1 of N-acetylglucosamine and N-acetylmuramic acid. As has been shown above, the free reducing group of the disaccharide is that of *N*-acetylmuramic acid. Since C-2 is occupied by the acetamido grouping, and C-3 by the lactic acid side chain (Strange & Kent, 1959), and assuming a pyranose ring to be present, only C-4 or C-6 remains available for attachment to the *N*-acetylglucosamine. It has been established for other *N*-acetylhexosamines that a substituent on C-4 precludes the formation of chromogens under the alkaline conditions of the Morgan–Elson reaction (Kuhn, Gauhe & Baer, 1954*b*). Since the disaccharide under discussion gave a Morgan–Elson reaction (Table 1) it might therefore be assumed that the linkage was (1:6). However, it was known (Perkins, 1960) that under the conditions of the reaction alkaline hydrolysis of glycosidic bonds could precede chromogen formation in lysozyme digests of cell walls, and that alkali would set free *N*-acetylglucosamine from the disaccharide (see below). It is not known whether *N*-acetylmuramic acid substituted on C-4 will undergo alkaline hydrolysis in this way. Nevertheless, it has been shown that when *N*-acetylhexosamine substituted on C-6 is treated with alkali, a chromogen can be formed to which the substituent is still attached (Kuhn *et al.* 1954*b*), a reaction which could not occur with a substituent on C-4. An attempt was therefore made to isolate, after alkaline treatment of the disaccharide, chromogenic material to which *N*-acetylglucosamine was still linked.

Samples of disaccharide were heated at 100° with triethylamine–carbonate buffer for various periods. After removal of the buffer the products were subjected to paper chromatography in butanol–acetic acid–water, and the unheated paper was sprayed with Ehrlich's reagent. With increasing time of heating in the alkali a slow-running chromogen first appeared, and then disappeared to be replaced by a fast-running chromogen ($R_{N\text{-acetylglucosamine}}$ 0·75 and 1·75 respectively). The latter chromogen is identical with that obtained from *N*-acetylglucosamine or *N*-acetylmuramic acid alone. The slow-running chromogen was separated from unchanged disaccharide and from liberated *N*-acetylglucosamine as follows.

Chromogenic material was prepared by heating a sample of disaccharide with triethylamine–carbonate buffer at 100° for 60 min. in a sealed ampoule. The material was dried in a vacuum desiccator and subjected to zone electrophoresis on washed paper at pH 6·5. In this way chromogenic material, which moved slightly towards the cathode, was easily separated from unchanged disaccharide, which moved several centimetres towards the anode. The compact chromogen band was eluted, dried, transferred to washed paper and run on a chromatogram in butanol–acetic acid–water overnight. Three bands were detectable on marker strips: free *N*-acetylglucosamine and, clearly separated from it, slow and fast-running chromogens as before.

The chromogen bands were eluted from the part of the paper which had not been sprayed, hydrolysed in 2N-HCl for 2 hr. at 105°, freed of HCl and again applied to a chromatogram and run in butanol–acetic acid–water with markers of muramic acid and glucosamine. The fast chromogen gave some weak ninhydrin-positive spots which corresponded to neither marker; the slow chromogen gave a clear spot of glucosamine, and in addition similar weak spots which seemed to derive from acid hydrolysis of free chromogen. Evidently the slow-running material consisted of chromogen derived from the *N*-acetylmuramic acid portion of the molecule which was still linked to unchanged *N*-acetylglucosamine. It is concluded therefore that the disaccharide isolated from lysozyme digests of cell wall has the composition 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid.

DISCUSSION

The work of Salton (1956), demonstrating the liberation by lysozyme from sensitive cell walls of an oligosaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid, has been confirmed and his suggestion that it might be a disaccharide has been substantiated. He also showed that free reducing groups not present in the isolated cell wall are progressively set free by the action of lysozyme. The present work shows that a considerable proportion (see also Perkins, 1960) of this reducing material is a disaccharide, the reducing group of which is that of *N*-acetylmuramic acid. Assuming that only one type of bond is attacked by lysozyme, then that bond must be a glycosidic link from *N*-acetylmuramic acid, possibly to *N*-acetylglucosamine, although there is no direct evidence for this at the moment. However, Berger & Weiser (1957) have shown that chitin, which is known to be a β-1:4-linked poly-*N*-acetylglucosamine, is attacked by egg-white lysozyme. It seems possible therefore that by analogy the link attacked in bacterial cell walls may be 4-*O*-β-(*N*-acetylmuramosyl)-*N*-acetylglucosamine.

At present it is not known whether an α- or a β-linkage is present in the 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid which has been isolated from the lysozyme digests of cell wall. It seems likely that this point might be elucidated by the use of α- or β-*N*-acetylglucosaminidases in conjunction with their specific inhibitors.

SUMMARY

1. An oligosaccharide has been isolated from lysozyme digests of the cell walls of *Micrococcus lysodeikticus*, and has been shown to contain only *N*-acetylglucosamine and *N*-acetylmuramic acid, thus confirming the work of Salton (1956).

2. Oxidation of this oligosaccharide with alkaline iodine solution, followed by acid hydrolysis, has shown that it is a disaccharide, the reducing terminus of which is *N*-acetylmuramic acid.

3. A study of the Morgan–Elson chromogens formed by heating the disaccharide with alkaline solutions has shown that its structure is 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid.

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Substances Reacting as Hexosamine and as *N*-Acetylhexosamine Liberated from Bacterial Cell Walls by Lysozyme

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It has long been known that egg-white lysozyme acts upon certain Gram-positive micro-organisms and their isolated cell walls to yield substances giving the Morgan & Elson (1934) reaction for *N*-acetylhexosamine (Epstein & Chain, 1940; Salton, 1956). Salton (1956) also demonstrated that at least some of the colour given in this reaction was due to the presence of an oligosaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid (see Perkins, 1960), but he did not give any quantitative figures for the amount of '*N*-acetylhexosamine' that he found. It has also been reported by Brumfitt, Wardlaw & Park (1958; cf. Table 1 in their paper) that material reacting in the Elson & Morgan (1933) test as 'glucosamine' is liberated by lysozyme from the cell walls of *Micrococcus lysodeikticus*. This result seemed to contradict the work of Ingram & Salton (1957), who treated lysozyme digests of bacterial cell walls with fluorodinitrobenzene and found no dinitrophenylglucosamine and only traces of dinitrophenylmuramic acid after acid hydrolysis of the product. In the work reported here these conflicting reports have been further examined. It has been found that direct measurements of '*N*-acetylhexosamine' and 'hexosamine' are an unreliable guide to the composition of lysozyme digests. In order to obtain quantitative information about the various fragments liberated by lysozyme, the mixture of compounds produced by the action of lysozyme on the cell walls of *M. lysodeikticus* has been fractionated and, after acid hydrolysis, the distribution of hexosamine between the fractions has been studied.

METHODS AND MATERIALS

Micro-organisms. The organisms used were *Micrococcus lysodeikticus* (N.C.T.C. 2665), *Bacillus subtilis* (N.C.T.C. 6346) and *Staphylococcus* strain 11 which was partially sensitive to lysozyme (Rogers & Perkins, 1959).

Preparation of cell walls. Cell walls were prepared essentially according to the method of Cummins & Harris (1956) as described by Perkins & Rogers (1959).

Action of lysozyme. Isolated cell walls were incubated at 37° with lysozyme (crystalline; The Armour Laboratories)

at a final concentration of 50 μg./ml. unless otherwise stated, at pH 6·1 in 0·1 M-buffer, which was either ammonium acetate or in some cases sodium and potassium phosphate, and the reaction was stopped by cooling to 0°. Estimations of 'hexosamine' and '*N*-acetylhexosamine' were performed on the supernatant solutions after centrifuging the lysozyme digests in the cold at 11 000 *g*. When lysozyme digests were to be employed for fractionation and other special procedures, incubation was continued for 3 hr., by which time the digestion was essentially complete. Soluble lysozyme digests were sometimes separated into diffusible and non-diffusible portions as described elsewhere (Perkins, 1960).

Estimation of hexosamine. Hexosamine was estimated by the procedure of Rondle & Morgan (1955), in some cases after preliminary separation of glucosamine and muramic acid as described by Perkins & Rogers (1959). In certain experiments designed to test the possibility that the 'hexosamine' colour obtained was an artifact, the concentration of acetylacetone in the reaction mixture and its pH value were varied as described by Immers & Vasseur (1950).

When the Rondle & Morgan (1955) procedure was carried out in the presence of material which had been treated previously with fluorodinitrobenzene as described below, it was necessary to correct for the absorption at 530 mμ which was due to the dinitrophenyl (DNP) derivatives. These compounds still absorb at 700 mμ, at which wavelength glucosamine in the Elson & Morgan (1933) test gives no absorption. Measurements were therefore made at a wavelength of 700 mμ, in order to obtain an indication of the amount of DNP compounds present. A correction factor was calculated and subtracted from the absorption value obtained at 530 mμ.

Estimation of N-acetylhexosamine. The colorimetric method of Reissig, Strominger & Leloir (1955), which employs borate buffer, pH 9·1, in its initial phase, and that of Aminoff, Morgan & Watkins (1952), which uses sodium carbonate, pH 10·8, were both employed in various stages of the work and comparisons were made between them.

Reaction of cell walls and lysozyme digests with fluorodinitrobenzene. Cell walls were treated with 1-fluoro-2:4-dinitrobenzene (FDNB) as described by Ingram & Salton (1957). Lysozyme digests of walls were prepared as described above and, after removal of the ammonium acetate buffer *in vacuo*, were allowed to react with FDNB, the solution being made alkaline by the addition of trimethylamine. After reaction for 2 hr. at room temperature the mixture was extracted repeatedly with benzene, and then the aqueous phase was dried in a vacuum desiccator over NaOH. The walls and digests after treatment with FDNB were hydrolysed for only 4 hr. in 4 N-HCl at 105° in sealed ampoules, so that destruction of acid-labile DNP derivatives, such as those of glycine, glucosamine and muramic acid, would be limited. In recovery experiments 55% of DNP-muramic acid and 45% of DNP-glucosamine remained after hydrolysis. Compounds present in these hydrolysates were identified on two-dimensional chromatograms, the buffered 2-methylbutan-2-ol (*tert.*-pentanol) solvent of Blackburn & Lowther (1951), followed by the phosphate buffer described by Levy (1954), being used. Marker spots of the DNP derivatives of all the relevant compounds were run in both dimensions.

In some cases lysozyme digests were treated under alkaline conditions similar to those used in the Rondle & Morgan (1955) procedure (i.e. heated for 20 min. at 100° in 0·167 N-Na_2CO_3 before reaction with FDNB). In these experiments the pH of the solution was then lowered with CO_2 to 8·2 before addition of ethanol and FDNB in the usual way. After acid hydrolysis the solution was extracted with ether, which would remove DNP-muramic acid along with non-basic DNP-amino acids. The DNP compounds remaining in the aqueous phase were subjected to zone electrophoresis in Na_2CO_3 solution (0·1 M), which was found to separate DNP-glucosamine, ϵ-DNP-lysine and dinitrophenol. This procedure avoided undue interference by the salt which was inevitably present under these conditions.

Acetylation of lysozyme digests of cell walls. In some experiments examination of lysozyme digests of cell walls by the Rondle & Morgan (1955) method for hexosamine and the Reissig *et al.* (1955) method for *N*-acetylhexosamine was preceded by acetylation procedures. Lysozyme digests of cell walls prepared as previously described were freed as far as possible from ammonium acetate, and the dry material was treated with acetic anhydride containing 10% (v/v) of pyridine, at 37° for 4 hr., a procedure which should acetylate free amino groups and possibly some hydroxyl groups. The product was extracted several times with ether and finally dried in a vacuum desiccator over NaOH and H_2SO_4. In order to remove any *O*-acetyl residues which had been introduced the acetylated material was allowed to stand overnight in 0·01 N-NaOH at room temperature.

Paper chromatography and zone electrophoresis. These techniques were performed as described by Perkins (1960).

Turbidity measurements. The turbidity of cell-wall suspensions was measured in the Hilger Spekker photoelectric absorptiometer.

Dinitrophenylglucosamine. This (m.p. 196°, uncorr.) was a crystalline sample prepared essentially according to the method of Annison, James & Morgan (1951).

Dinitrophenylmuramic acid. This was prepared by the action of fluorodinitrobenzene on muramic acid which had been isolated from cell walls. It was isolated from a paper chromatogram, and the solution used as a marker and in recovery experiments from acid hydrolysates.

RESULTS

Lysozyme digestion of cell walls and the liberation of materials reacting as 'hexosamine' and 'N-acetylhexosamine'. Isolated cell walls of *M. lysodeikticus* were incubated with lysozyme (10 μg./ml.) at 37° and samples were taken at intervals for measurement of turbidity, '*N*-acetylhexosamine' by the procedure of Reissig *et al.* (1955) and 'hexosamine' by the method of Rondle & Morgan (1955). The result of a typical experiment is given in Fig. 1, which shows that the amount of apparent *N*-acetylhexosamine liberated and estimated under these conditions represents a very small proportion of the wall as a whole. Perkins & Rogers (1959) have indicated that the wall contains about 15% of *N*-acetylglucosamine, and an approximately equimolecular proportion of *N*-acetylmuramic acid, so that the maximal liberation of *N*-acetylhexosamine

according to Fig. 1 would correspond to only about 0·7 % of the *N*-acetylglucosamine present in the wall. On the other hand, the amount of 'hexosamine' estimated is equal to about ten times the '*N*-acetylhexosamine' and is in good agreement with the value found by Brumfitt *et al.* (1958). In parallel experiments conducted with the isolated cell walls of *B. subtilis* and a strain of *Staphylococcus* (strain 11), a similar relationship was observed between the amounts of 'hexosamine' and '*N*-acetylhexosamine' liberated. In the Rondle & Morgan (1955) test glucosamine gives an absorption maximum at 530 mμ, whereas muramic acid produces a colour with a maximum at 510 mμ (Crumpton, 1959). The 'hexosamine' colour given by lysozyme digests of cell walls has an absorption maximum at about 525 mμ, that is, not far removed from the glucosamine peak, and quite unlike that of muramic acid (Fig. 2). Since it appeared possible that lysozyme had liberated from cell walls some kind of substituted hexosamine having a free amino group, which would react in the Rondle & Morgan test, an attempt was made to identify any such free amino groups.

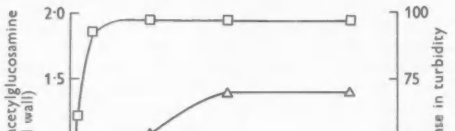

Fig. 1. Action of lysozyme on the isolated cell walls of *M. lysodeikticus*. Reduction in turbidity and liberation of materials reacting as 'glucosamine' (Rondle & Morgan, 1955) and '*N*-acetylglucosamine' (Reissig *et al.* 1955). □, Decrease in turbidity; △, 'glucosamine'; ○, '*N*-acetylglucosamine'.

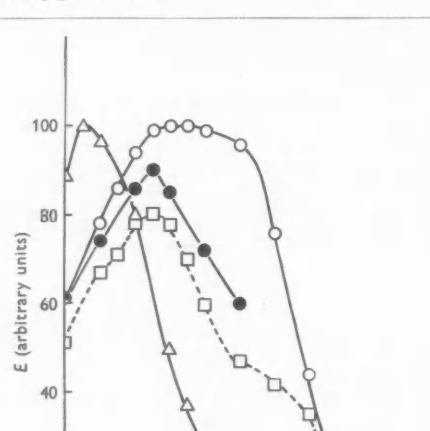

Fig. 2. Absorption curves in hexosamine test (Rondle & Morgan, 1955). △, Muramic acid; ○, glucosamine; □, 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid; ●, lysozyme digest of isolated cell walls of *M. lysodeikticus*.

Examination for free amino groups. The isolated cell walls of *M. lysodeikticus* were treated with FDNB and, after acid hydrolysis, the only DNP derivatives which could be found by two-dimensional paper chromatography were DNP-alanine (in small yield) and ε-DNP-lysine (in large yield), a result which confirms that of Ingram & Salton (1957). When the cell walls were first digested with lysozyme and then treated in the same way, the result was qualitatively the same; in contrast with the findings of Ingram & Salton (1957) not even a trace of DNP-muramic acid could be detected. From the results given above it seemed possible that the amino groups of glucosamine or muramic acid in lysozyme digests of cell walls might be set free under the alkaline conditions of the Rondle & Morgan (1955) hexosamine test. Accordingly, digests were heated under these conditions for 20 min. at 100°, before being treated with FDNB. After acid hydrolysis the ether-soluble derivatives were examined by paper chromatography and the water-soluble portion by zone electrophoresis in Na_2CO_3 buffer. Once more the DNP derivatives of glucosamine and muramic acid could not be found. It seemed therefore that the explanation for the 'hexosamine' reaction observed in lysozyme digests of cell walls must be sought elsewhere.

Investigation of 'hexosamine' colour reaction. The best-known artifact colour in the Elson & Morgan (1933) reaction is given by mixtures of glucose with glycine or lysine (Sideris, Young & Krauss, 1938; Immers & Vasseur, 1950). The latter authors showed that, compared with the colour given by glucosamine, the artifact colour was only slightly diminished by decreasing the amount of acetylacetone used in the alkaline reagent from 2 % (v/v) to 0·75 % (v/v). On the other hand, when a higher concentration of acetylacetone was used at a lower pH value, it was the development of artifact colour

Table 1. *Variation of colour development in the Rondle & Morgan (1955) test with altered acetylacetone concentration*

Acetylacetone concentration in alkaline reagent (%, v/v)	Alkaline buffer used	Extinction at 530 mμ (1 cm. cell)	
		25 μg. of glucosamine hydrochloride	Lysozyme digest of 1·3 mg. of cell wall
0·75	Na_2CO_3 (0·5 N)	0·081	0·284
2·0	Na_2CO_3 (0·5 N)	0·245	0·292
5·0	Na_2CO_3–$NaHCO_3$ (M), pH 9·5	0·214	0·210

which was adversely affected. Furthermore, when mixtures of glucose and glycine or lysine were treated by the Elson & Morgan (1933) procedure, but with the acetylacetone omitted, then unlike glucosamine they gave a coloured product with maximal absorption at about 560 mμ.

In the present experiments cell wall isolated from *M. lysodeikticus* was incubated with lysozyme (final concentration 50 μg./ml.) at 37° in phosphate buffer (0·1 M, pH 6·5) for 2 hr. Samples of digest were treated by the Rondle & Morgan procedure, except that the acetylacetone concentration and the pH value were varied in the manner described by Immers & Vasseur (1950). The results given in Table 1 show that the colour developed by lysozyme digests was relatively unaffected by the changed conditions, whereas lowered acetylacetone concentration produced a considerable diminution in colour yield from glucosamine. In a similar experiment acetylacetone was omitted altogether, and a colour appeared which was shown within the range 500–600 mμ to have a single absorption maximum at 555 mμ. In these respects the behaviour of the digests resembled that described by Immers & Vasseur (1950) for mixtures of glucose and lysine. It seemed possible therefore that the colour given in the Rondle & Morgan test by lysozyme digest of cell walls might have been an artifact due to the free ε-amino groups of lysine (known to be present from the work with FDNB described above) and the reducing groups of some sugar. The sugar need not have been glucose, since as mentioned earlier the same effect could be observed with the cell walls of *Staphylococcus* (strain 11), which had been shown on hydrolysis to contain glucosamine and muramic acid as their only carbohydrate components.

Reduction of production of artifact colour in the Elson & Morgan (1933) test. It was found that if lysozyme digests of cell walls were treated in such a way as to mask the free amino groups of lysine, then the colour development in the Rondle & Morgan test could be reduced by 60 %. Cell walls of *M. lysodeikticus* were digested with lysozyme in ammonium acetate buffer as previously described, and the whole digest was freeze-dried under high vacuum. One sample was treated with FDNB in order to prepare the DNP derivative of the ε-amino groups of lysine, and another was acetylated with acetic anhydride and pyridine. The latter procedure was followed by treatment with NaOH (0·01 N) to remove any *O*-acetyl groups which might have been introduced. Samples of the products were then treated by the Rondle & Morgan (1955) hexosamine procedure, and also by the Reissig *et al.* (1955) method for *N*-acetylhexosamine, with the result shown in Table 2. There was a slight reduction in the amount of apparent *N*-acetylhexosamine estimated, but the 'hexosamine' colour produced could be decreased to about 40 % of its initial value. This result suggests that the ε-amino group of lysine, known to be the only free amino group present in any quantity in these digests, is involved in the production of a large proportion of the colour obtained in the Rondle & Morgan test.

Table 2. *Effect of masking the ε-amino groups of lysine on the reaction given by lysozyme digests of cell walls in the N-acetylhexosamine and hexosamine tests*

Treatment of lysozyme digest of cell walls	Percentage of cell wall estimated	
	As *N*-acetylglucosamine*	As glucosamine†
None	0·178	1·62
Acetylated (20°; 50 min.)	0·163	0·99
Acetylated (37°; 4 hr.)	0·142	0·63
Fluorodinitrobenzene	—	0·88

* Reissig, Strominger & Leloir (1955).
† Rondle & Morgan (1955).

Production of 'hexosamine' colour by a disaccharide isolated from lysozyme digests of cell walls. The preceding paper (Perkins, 1960) describes the isolation from lysozyme digests of the cell walls of *M. lysodeikticus* of a disaccharide characterized as 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid. Although under conditions of the *N*-acetylhexosamine tests employed this material yields a colour characteristic of *N*-acetylamino sugars, when treated by the Rondle & Morgan procedure it also yields a colour with an absorption maximum at 525 mμ (Fig. 2), presumably due to complex reactions caused by the alkaline treatment (see

below). It seems probable therefore that the presence of this material may have accounted for the residual colour production, which could not be eliminated by masking the free amino groups of lysine (Table 2).

N-Acetylhexosamine reaction of lysozyme digests of cell walls. In their work upon blood-group substances, which like bacterial cell walls contain bound *N*-acetylamino sugars, Aminoff *et al.* (1952) showed that when these substances were heated with Na_2CO_3 solution under the conditions of their *N*-acetylhexosamine test, the amount of colour produced on addition of Ehrlich's reagent varied with the time of heating. Maximum colour production was not necessarily obtained after the same time interval as that required for the free *N*-acetylhexosamines. Such differences are related to the position of substitution of the *N*-acetylamino sugar which is giving the reaction (see Buchanan, Crumpton & Morgan, 1957). The *N*-acetylhexosamine reaction of lysozyme digests of cell walls behaved similarly to that of the blood-group substances when tested under varying conditions of treatment with alkali.

Lysozyme digests of the isolated cell walls of *M. lysodeikticus* were prepared as described earlier. In the particular experiment discussed here the diffusible portion of the digest was employed, but whole digests gave essentially similar results. Samples of the diffusible material were heated at 100° for various times under the conditions of Reissig *et al.* (1955), who used borate buffer at about pH 9·0, or those of Aminoff *et al.* (1952), who used carbonate, pH 10·8. After the addition of the acetic acid solution containing *p*-dimethylaminobenzaldehyde in the usual way, the light absorption was measured at 585 mμ. The results given in Fig. 3 are expressed in terms of the apparent amount of *N*-acetylglucosamine present, i.e. the absorption at 585 mμ of each sample is compared with that given by *N*-acetylglucosamine heated in the corresponding conditions for the time necessary to give maximal colour yield. In most cases the absorption curve of the final solution was essentially the same as that given by *N*-acetylglucosamine, but, after heating for 60 min. in the carbonate solution, the peak at 585 mμ had disappeared and a single peak at 555 mμ had arisen. This latter peak resembled the one obtained in the Rondle & Morgan test without acetylacetone, described above.

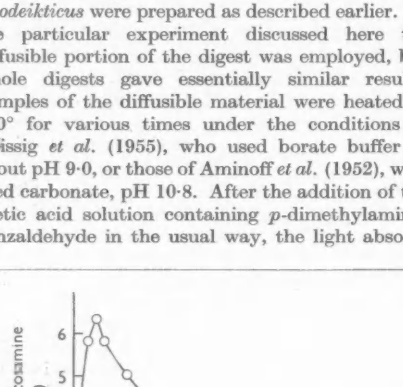

Fig. 3. Development of colour in *N*-acetylhexosamine tests given by the diffusible fraction of a lysozyme digest of isolated cell walls (*M. lysodeikticus*). △, Borate, pH 9·1 (Reissig *et al.* 1955); ○, carbonate, pH 10·8 (Aminoff, Morgan & Watkins, 1952).

Evidently the higher pH value of the carbonate solution used in the method of Aminoff *et al.* (1952) facilitated the formation of chromogens from the wall digests, probably by accelerated alkaline hydrolysis. It is apparent that the quantity of '*N*-acetylhexosamine' measured in lysozyme digests of cell walls is largely a function of the conditions used in the test, and is no direct indication of the number of *N*-acetylhexosamine-reducing groups set free by the enzyme.

Distribution of hexosamine amongst fractions of a lysozyme digest of cell walls. Although Salton (1956) discussed the presence of materials reacting as *N*-acetylhexosamine in the diffusible and non-diffusible fractions of lysozyme digests of cell walls, so far no quantitative figures have been available for the distribution of these components. From the foregoing results it was evident that the ordinary reactions for hexosamine or *N*-acetylhexosamine were not likely to provide reliable information on this point. It was therefore decided to separate the wall digests into various fractions, and to estimate the total amount of hexosamine present in each fraction after acid hydrolysis.

A lysozyme digest of the cell walls of *M. lysodeikticus* was separated into diffusible and non-diffusible fractions as described by Perkins (1960). Each fraction was freeze-dried in a high vacuum, but it was difficult to remove ammonium acetate completely from the diffusible fraction. The percentage of the whole material in this portion was therefore taken as the difference between the original weight and that of the non-diffusible material. When samples of the diffusible material were run on paper chromatograms in butanol–pyridine–water, material reacting with aniline phthalate and giving a purple with the hexosamine spray reagent (Partridge, 1948) could be found in two regions only, one running from the origin (fraction A), and the other at $R_{N\text{-acetylglucosamine}}$ 0·85. The latter spot was found to be a disaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid corresponding to the oligosaccharide described by Salton (1956). It is discussed in detail in the

preceding paper (Perkins, 1960). A sample of the diffusible fraction of a lysozyme digest was separated in this way on washed paper (Hanes, Hird & Isherwood, 1952). The two regions were eluted and samples were hydrolysed in acid before separation and estimation of glucosamine and muramic acid. Other regions of the chromatogram were not found to contain any hexosamine, and it was therefore assumed that the whole of the hexosamine of the diffusible material was contained in these two fractions. In all fractions studied the glucosamine was accompanied by an approximately equimolecular proportion of muramic acid. The distribution of glucosamine among the various fractions is shown in Table 3. Lysozyme converts about 20% of the cell-wall amino sugars into the disaccharide and about 50% into other compounds diffusible through cellophan. Neither portion of the diffusible fraction was found to contain any of the glucose present in the whole cell wall.

Composition of fraction A. The portion of the diffusible material which moved slowly on butanol–pyridine–water chromatograms (fraction A) was examined further. After zone electrophoresis in acetic acid (2N), three well-defined bands were observed (one moved 0·8 cm. towards the anode and the others 4·8 and 6·8 cm. towards the cathode), all of which reacted to a greater or lesser extent with ninhydrin, aniline phthalate and *N*-acetylhexosamine reagent. Samples of these components eluted from washed paper were hydrolysed in 4N-HCl for 4 hr. and the hydrolysates were examined by two-dimensional paper chromatography in butanol–pyridine–water and butanol–acetic acid–water. Each fraction was composed of all the components of whole cell walls with the exception of glucose, which was found in a separate experiment to be absent. The anionic material contained a preponderance of glucosamine and muramic acid with only small amounts of the four amino acids and hence may not have been homogeneous, but no accurate quantitative analysis of the fractions has yet been made. Apart from the disaccharide therefore the diffusible fraction of lysozyme digests of cell walls contains compounds in which the amino sugars are bound to amino acids.

Table 3. *Distribution of bound glucosamine in fractions of a lysozyme digest of cell walls of* Micrococcus lysodeikticus

Fraction of digest		Percentage (by wt.) of whole cell	Glucosamine (% of total)
Non-diffusible		56·5	28·5
Diffusible	Disaccharide	—	19·9
	Remainder	—	51·6
	Total	43·5	71·5

DISCUSSION

The action of lysozyme on certain bacterial cell walls was studied by Salton (1956), who described the liberation of materials giving the Morgan & Elson (1934) reaction for *N*-acetylhexosamines, although he did not give any quantitative estimate of the amount. Although the exact nature of the groups set free by lysozyme was not known, he was able to show that some proportion of the *N*-acetylhexosamine reaction he obtained was due to an oligosaccharide, possibly a disaccharide, of *N*-acetylglucosamine and *N*-acetylmuramic acid.

In the present work it has been shown that the quantity of reactive *N*-acetylhexosamine groups measured in the usual tests is no direct indication of the number of such groups present in the digests, since the intensity of colour produced is a function of the alkalinity of the reaction mixture and the time of heating. The Elson & Morgan (1933) reaction for hexosamine, as measured by Brumfitt *et al.* (1958), has been shown not to be due to the presence of non-acetylated hexosamine end groups. In all probability it is an artifact produced partly by the simultaneous presence of carbohydrate-reducing groups and the free ϵ-amino groups of lysine, and partly by alkaline decomposition of the chromogen initially formed from *N*-acetylmuramic acid end groups (see also Perkins, 1960). It is not possible therefore to deduce anything about the proportion of the amino sugar end groups of cell walls set free by lysozyme from the amount of colour developed either in the Elson–Morgan (hexosamine) or in the Morgan–Elson (*N*-acetylhexosamine) reaction.

Brumfitt *et al.* (1958) proposed a simple repeating unit as the structural basis of the cell walls of lysozyme-sensitive organisms, and suggested that C-1 of *N*-acetylmuramic acid was set free by lysozyme. Their evidence for the latter assertion was based on the relative amount of colour developed in *N*-acetylhexosamine tests in the presence of borate as opposed to carbonate (Park, 1956). As Reissig *et al.* (1955) and Cifonelli & Dorfman (1958) have shown, the colour yield of 3-substituted *N*-acetylhexosamines such as *N*-acetylmuramic acid is unaffected by the use of borate, which with *N*-acetylglucosamine itself gives a greatly enhanced colour value. It can be seen from Fig. 3, however, that interpretation of the amount of colour yielded by lysozyme digests of cell walls in these tests must necessarily be difficult. Nevertheless, the evidence given by Perkins (1960) shows that lysozyme does indeed liberate the C-1 of *N*-acetylmuramic acid, which at least in some parts of the wall structure is linked by C-6 to C-1 of *N*-acetylglucosamine.

On the basis of their experiments Brumfitt *et al.* (1958) suggested that lysozyme acts on only about

one in ten of the *N*-acetylmuramic acid glycosidic bonds in their proposed repeating structure. It can be seen from Table 3 that lysozyme does not set free from the wall a number of essentially similar fragments, but rather that the product is a heterogeneous mixture which can be divided into three fractions.

The smallest molecule found in the digests is the disaccharide, 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid. Unless the enzyme causes extensive transglycosidation, about 20% of the hexosamines of the wall must occur as this disaccharide, in which the muramic acid carboxyl group is not linked to amino acids. The reducing group of the muramic acid may be linked either to the *N*-acetylglucosamine part of a second molecule of disaccharide, or to some other unknown point in the wall structure.

The second fraction, forming the remainder of the diffusible portion of the wall digests, consists of larger molecules in which the amino sugars are linked to amino acids. It is probable that in these compounds the amino acids are in the form of peptide chains bound in amide linkage to the carboxyl group of muramic acid, since it has been possible to isolate this type of compound from partial acid hydrolysates of the cell wall of *M. lysodeikticus* (Perkins & Rogers, 1959). The fact that this second group of products from lysozyme digestion contains at least three distinct fragments separable by electrophoresis suggests a heterogeneity in the wall from which they come. Probably the peptide chains attached to the muramic acid do not all have the same composition.

The third fraction comprises the non-diffusible portion of the digest, which is apparently resistant to further attack by lysozyme, and contains the bulk of the glucose present in the wall. It seems possible either that the lysozyme-sensitive linkage does not occur in this fraction, or that attack by the enzyme is inhibited by the presence of some other substituent on one of the hexosamine moieties, rather in the manner that the presence of the *O*-acetyl group can be shown to inhibit its action (Brumfitt *et al.* 1958).

Assuming that only one type of bond is split by lysozyme, the variability in composition of the fragments which can be found in lysozyme digests of the walls of *M. lysodeikticus* suggests once more that the mucopeptide of these structures may be heterogeneous, as was indicated by Rogers & Perkins (1959).

SUMMARY

1. Lysozyme digests of the cell walls of *Micrococcus lysodeikticus* were prepared, and their reactions in the Elson–Morgan (hexosamine) and Morgan–Elson (*N*-acetylhexosamine) tests examined.

2. The Elson–Morgan reaction given for 'hexosamine' was shown to be an artifact, since no free amino sugar amino groups could be demonstrated.

3. The amount of colour given by lysozyme digests in the *N*-acetylhexosamine reaction depended upon the pH of the alkaline solution used in the Morgan–Elson test and upon the time of heating.

4. Cell-wall digests were fractionated, and the distribution of wall components amongst the fractions was studied: 20% of the hexosamine appeared as 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid and 50% as diffusible material in which the amino sugars were bound to amino acids.

5. The significance of these results in connexion with cell-wall structure has been discussed.

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Biological Synthesis of L-Ascorbic Acid in Animal Tissues: Conversion of L-Gulonolactone into L-Ascorbic Acid

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The enzyme system catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid in animal tissues has been previously reported to be entirely located in the microsomal fractions of rat- and goat-liver homogenates (Burns, Peyser & Moltz, 1956; Chatterjee, Ghosh, Ghosh & Guha, 1957*a*, *b*; 1958*a*, *b*). The kidney tissue has been found to be the site of this conversion in amphibian, reptilian and some of the avian species examined (Roy & Guha, 1958), and in chick kidney the enzyme system concerned has also been found to be located in the microsomes. Isherwood (1953) stated that mitochondria from rat liver catalyse the transformation of L-gulonolactone into L-ascorbic acid. However, our experiments indicate that the observed activity of mitochondria is probably due to their contamination by microsomes. Potassium cyanide greatly accelerates this synthesis from D-glucuronolactone but the synthesis from L-gulonolactone does not require cyanide or any other added factor (Chatterjee *et al.* 1958*b*). Subsequently the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes has been found to be greatly accelerated by boiled rat-liver supernatant or a metal-binding agent such as sodium pyrophosphate, $\alpha\alpha'$-dipyridal and 8-hydroxyquinoline (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1958*c*). The microsomal enzyme concerned in the oxidation of L-gulonolactone to L-ascorbic acid is inhibited by heavy-metal ions (Hg^{2+}, Cu^{2+} and Zn^{2+}) and reversibly by *p*-chloromercuribenzoate, indicating the involvement of some essential thiol groups in the enzyme (Chatterjee *et al.* 1958*c*).

It has already been reported that the microsomes can convert only the lactone forms of the precursors, namely D-glucuronolactone and L-gulonolactone. The sodium salts of the corresponding free acids are not acted on (Chatterjee *et al.* 1957*b*; 1958*a*, *b*). The soluble supernatant has been found to inhibit the rate of conversion of L-gulonolactone into L-ascorbic acid to a great extent and, though L-gulonate is not converted by the microsomes alone, it leads to the formation of L-ascorbic acid when soluble supernatant is added to the system. The factor in the supernatant which inhibits the rate of microsomal synthesis of ascorbic acid from L-gulonolactone but, curiously, helps in the microsomal synthesis from L-gulonate as the substrate has been traced to a strong lactonase, which has been termed gulonolactonase in this paper (Chatterjee *et al.* 1958*b*; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*a*). This enzyme has been partially purified and characterized as an enzyme which acts reversibly by hydrolysing gulonolactone to the free acid and also lactonizing the free acid to gulonolactone, the equilibrium being more favourable for the formation of the free acid. When the lactone is used as the substrate, its conversion into ascorbic acid is diminished in the presence of the supernatant owing to this enzyme hydrolysing the lactone. On the other hand, when gulonate is used as the substrate, a small part of it is converted into lactone and this lactone is then acted on by the microsomal enzyme producing ascorbic acid (Chatterjee *et al.* 1959*a*). Therefore it is the gulonolactone and not the gulonate which is specifically acted on by the enzymes concerned, for the formation of ascorbic acid. In guinea-pig liver the gulonolactonase has been found to be present but L-gulono-oxidase, the enzyme catalysing the oxidation of L-gulonolactone to L-ascorbic acid, has been found missing (Chatterjee *et al.* 1958*c*). Recently a 30-fold concentrated soluble preparation of goat-liver microsomal enzyme (L-gulono-oxidase), converting L-gulonolactone into L-ascorbic acid, has been obtained (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*b*). The soluble enzyme acts only on the lactone and not on the free acid, again confirming the earlier observation from this Laboratory (Chatterjee *et al.* 1957*b*; 1958*a*, *b*) that the lactone and not the free acid is the precursor of ascorbic acid.

With this and other information in hand a detailed investigation was carried out on the mechanism of synthesis of ascorbic acid in animal tissues. This paper deals with the conversion of L-gulonolactone into L-ascorbic acid by liver microsomes of the rat and the goat and kidney microsomes of the chick as well as with some other features of the reaction mechanism involved. Since the publication of the above-mentioned papers from this Laboratory, a paper by Kanfer, Burns & Ashwell (1959) has appeared which confirms our previous observations on the specificity of the

lactone form of the precursor for the biosynthesis of ascorbic acid (Chatterjee *et al.* 1957*a*, *b*; 1958*a*, *b*; 1959*a*).

EXPERIMENTAL

Materials

Preparation of microsomes. The tissue extract (liver from rat and goat and kidney from chick), freed from the nuclear and mitochondrial fractions and containing the microsomes and the soluble supernatant, was prepared as described before (Chatterjee *et al.* 1958*b*). The extract was centrifuged at 88 700 *g* for 1 hr. in a Spinco model L preparative ultracentrifuge with rotor type no. 30. The supernatant was drained off, separated from the fatty layer and again centrifuged for 1 hr. to sediment any microsomal matter present. The clear supernatant layer which stood at the top of a fluffy layer was drawn off and kept aside and is referred to in the text as the soluble supernatant. The sedimented microsomes, after the supernatant had been completely drained out and the walls of the centrifuge tubes wiped with filter paper, were pooled, washed once with iso-osmotic sucrose solution by recentrifuging at 88 700 *g* for 2 hr. and dispersed in cold iso-osmotic sucrose in a Waring Blendor for 15 sec. The concentration of the dispersion was so adjusted that 1 ml. of the dispersion contained an amount of microsomes equivalent to 1 g. of wet tissue. The temperature throughout the procedure was kept at 0–2°. When potassium chloride solution was used in place of the sucrose solution as the homogenizing medium which was done especially for xylulose determination, the concentration of potassium chloride was 150 mM and the nuclear and mitochondrial fractions were sedimented at 4000 *g*, other procedures being the same as those carried out with the sucrose solution.

The dispersed rat-liver microsomes were generally stored at 0° in the lyophilized condition in sealed tubes of 5 ml. or 10 ml. capacity under vacuum. In this condition they were stable at least for a year without any loss of activity. Goat-liver microsomes, however, could be stored in the frozen state (−5°) without lyophilization for months together, but with rat liver the activity of the frozen microsomes fell gradually after 4–5 days.

Preparation of gulonolactonase. The soluble supernatant was heated at 50° for 30 min. with constant stirring, centrifuged and the clear centrifugate was fractionally precipitated with saturated ammonium sulphate at pH 6·0. The protein fraction at 50–80% saturation of the salt was collected and dissolved in water. This enzyme preparation acted reversibly on gulonolactone, hydrolysing it to the free acid as well as converting the free acid into the lactone and is referred to in the text as gulonolactonase. This enzyme is probably identical with the aldonolactonase described by Winkelman & Lehninger (1958).

Boiled supernatant. The soluble supernatant was heated at 100° on a water bath for 5 min., with constant stirring, and centrifuged. The clear pale-yellow centrifugate is referred to in the text as the boiled supernatant.

L-Gulonic dehydrogenase. The protein fraction of rat-liver soluble supernatant obtained at 30–50% saturation with ammonium sulphate at pH 7·4, followed by dialysis, is referred to in the text as L-gulonic dehydrogenase.

Chemicals. L-Gulonolactone was prepared by a modified method of Wolfrom & Anno (1952), Dowex 50 being used as the cation-exchange resin. A sample was also kindly supplied by Dr S. Ishikawa, Tokyo University, Japan. Sodium gulonate was prepared as by Chatterjee *et al.* (1958*b*). Sodium L-*xylo*hexulonate (sodium 2-keto-L-gulonate) was prepared from the 2:3:4:6 diacetone derivative of L-*xylo*hexulonic acid (2-keto-L-gulonic acid) following the method of Reichstein & Grüssner (1934). The diacetone derivative was a gift from Takeda Pharmaceutical Industries Ltd., Osaka, Japan, who also kindly supplied samples of flavinadenine dinucleotide (FAD), 93% pure and flavin mononucleotide (FMN). Riboflavin monosulphate was kindly supplied by Dr K. Yagi, Nagoya University, Japan. A sample of D-xylulose was obtained through the courtesy of Dr G. Ashwell, Bethesda, Md., U.S.A. Diphosphopyridine nucleotides (DPN and DPNH, over 90% purity) were from Sigma Chemical Co., U.S.A.; pancreatic lipase (steapsin) was from Nutritional Biochemical Corp., U.S.A. and ribonuclease from the Worthington Biochemical Corp., U.S.A.

Solutions of potassium cyanide, sodium pyrophosphate and other relatively unstable compounds used in the various experiments were freshly made and neutralized where necessary before addition to the reaction media.

Methods

Lyophilization. Lyophilization was carried out in an Edwards lyophilizer (Speedivac model 30P Centrifugal freeze-drier).

Incubation media. Unless otherwise mentioned, the test system contained 20 mM-sodium phosphate buffer, pH 7·4, 5 mM-L-gulonolactone or sodium L-gulonate and 0·25 ml. of microsomal dispersion (equivalent to 250 mg. of wet tissue). The total volume was 2·5 ml. and the digest was incubated in air at 37° for 1·5 hr.

Estimation of ascorbic acid. Ascorbic acid was identified and estimated by the procedure described before (Chatterjee *et al.* 1958*b*).

Identification of L-gulonolactone. L-Gulonolactone was identified as the hydroxamic acid derivative. The identification of L-gulonolactone formed from L-gulonate by the action of gulonolactonase was carried out by the following procedure.

L-Gulonate (10 mM) was incubated for 2 hr. with gulonolactonase (1 mg. equivalent of protein) in 20 mM-sodium phosphate buffer, pH 7·0, containing 0·5 ml. of neutral 2M-hydroxylamine. The total volume was 2 ml. At the end of incubation 1 ml. of the mixture was mixed with 1 ml. of neutral 2M-hydroxylamine and allowed to stand for 30 min. The hydroxamic acid derivative of L-gulonolactone formed was extracted with 20 ml. of hot absolute ethanol, centrifuged and the protein-free clear centrifugate was evaporated under suction at 40° to about 3 ml. The solution was evaporated to dryness at 0° in a vacuum desiccator and the residue taken up with 0·25 ml. of water. A portion of it (50 μl.) was applied on paper for chromatographic separation with formic acid–water (4 : 1, v/v) as the irrigating solvent. A parallel run was given with an authentic sample of L-gulonolactone. After development for 4 hr. the paper was air-dried and sprayed with a 0·5% $FeCl_3,6H_2O$ in 95% ethanol containing mM-HCl. A single spot corresponding to the hydroxamic acid derivative of authentic L-gulonolactone, R_F 0·8, was obtained. No lactone formation could be detected by incubating L-gulonate with boiled gulonolactonase under identical conditions.

Estimation of lactone. This was carried out by an adaptation of the hydroxamic acid procedure of Lipmann & Tuttle (1945). In the presence of tissue the modified method of Eisenberg & Field (1956) was used. Since the rate of hydrolysis of the lactone to the free acid is several times greater than the formation of lactone from free acid, estimation of the lactone formed from sodium L-gulonate by the action of gulonolactonase was carried out by using hydroxylamine as a trapping agent in the system, as mentioned above under 'Identification of L-gulonolactone'.

Estimation of DPNH. DPNH was estimated by measuring the extinction at 340 mμ in a Beckman model DU Spectrophotometer.

Identification and estimation of L-xylulose. L-Xylulose was identified by paper chromatography by the method of Touster, Hutcheson & Rice (1955), D-xylulose being used as the standard. The estimation of L-xylulose was carried out according to the method of Kulka (1956), a sample of authentic D-xylulose being taken as the standard.

Estimation of protein. Protein was estimated by the Biuret method described before (Chatterjee *et al.* 1958*b*), checked frequently by the micro-Kjeldahl method.

RESULTS

Microsomal conversion of L-gulonolactone into L-ascorbic acid by different species. It has already been reported that the enzyme catalysing the conversion of L-gulonolactone into L-ascorbic acid resides entirely in the microsomes (Chatterjee *et al.* 1958*a*). Table 1 shows the conversion of L-gulonolactone into L-ascorbic acid by liver or kidney microsomes from the different species studied.

Table 1. *Microsomal conversion of L-gulonolactone into L-ascorbic acid by different species*

Conditions are given in the Methods section.

Species	Tissue	Ascorbic acid synthesized (μmole)
Rat	Liver	0·80
Goat	Liver	2·75
Chick	Kidney	0·85

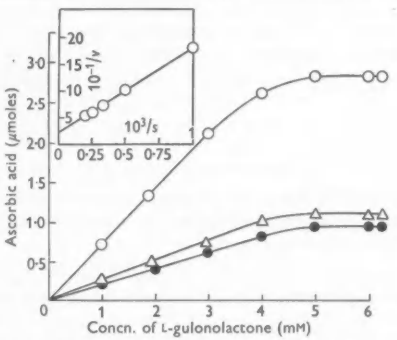

Fig. 1. Rate of formation of L-ascorbic acid as affected by substrate concentration. ○, Goat-liver microsomes; △, chick-kidney microsomes; ●, rat-liver microsomes. Inset figure represents $1/v \times 1/s$ for L-gulonolactone with goat-liver microsomes, where s is L-gulonolactone in moles/l. and v is μmoles of ascorbic acid formed/hr.

Kinetics of the enzymic conversion of L-gulonolactone into L-ascorbic acid. The Michaelis constants for L-gulonolactone with microsomal enzymes from goat, chick and rat, as calculated from Fig. 1, are 16, 10 and 10·4 mM, respectively. With a given concentration of the substrate the rate of synthesis increases proportionally with increasing concentration of the microsomes up to a certain point, beyond which no such proportionality is noticed (Fig. 2). The maximum formation of ascorbic acid takes place after an incubation period of 1·5 hr. (Fig. 3).

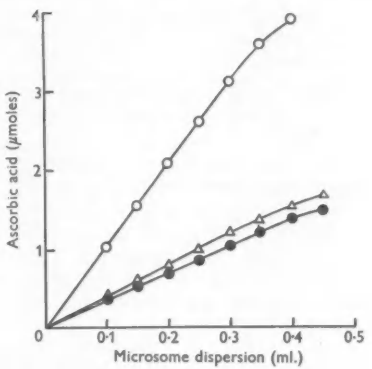

Fig. 2. Rate of formation of L-ascorbic acid as affected by enzyme concentration. Conditions are given in the Methods section. ○, Goat-liver microsomes; △, chick-kidney microsomes; ●, rat-liver microsomes.

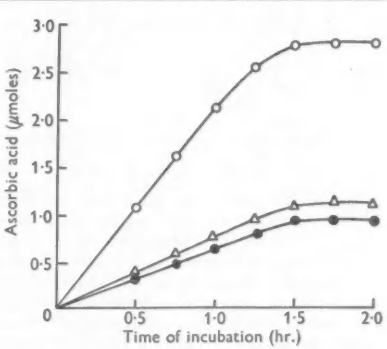

Fig. 3. Rate of formation of L-ascorbic acid from L-gulonolactone by microsomes. Conditions are given in the Methods section. ○, Goat-liver microsomes; △, chick-kidney microsomes; ●, rat-liver microsomes.

Acclerating effect of certain metal-binding agents on the conversion of L-*gulonolactone into* L-*ascorbic acid by rat-liver microsomes.* Certain metal-binding agents, i.e. sodium pyrophosphate, αα′-dipyridyl and 8-hydroxyquinoline, have been found to accelerate the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes (Table 2).

Table 2. *Effect of metal-binding agents on the conversion of* L-*gulonolactone into* L-*ascorbic acid by rat-liver microsomes*

Conditions are given in the Methods section.

Addition	Concn. (mM)	Ascorbic acid synthesized (μmoles)
None	—	0·82
Sodium pyrophosphate	5	1·40
αα′-Dipyridyl	2	1·35
8-Hydroxyquinoline	0·5	1·30

Table 3. *Effect of boiled supernatant on the conversion of* L-*gulonolactone into* L-*ascorbic acid by rat-liver microsomes*

Concentration of boiled supernatant used was 100 mg. equivalent of wet tissue. Other conditions are given in the Methods section.

Source of boiled supernatant	Ascorbic acid synthesized (μmoles)
None	0·80
Rat liver	1·45
Goat liver	1·20
Chick kidney	1·35
Guinea-pig liver	1·40

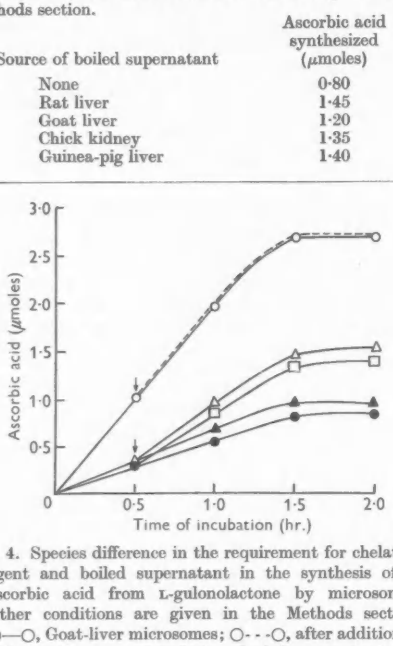

Fig. 4. Species difference in the requirement for chelating agent and boiled supernatant in the synthesis of L-ascorbic acid from L-gulonolactone by microsomes. Other conditions are given in the Methods section. ○—○, Goat-liver microsomes; ○- - -○, after addition of either sodium pyrophosphate or boiled supernatant. ▲, Chick-kidney microsomes; △, after addition of sodium pyrophosphate or boiled supernatant. ●, Rat-liver microsomes; □, after addition of sodium pyrophosphate or boiled supernatant. Arrows indicate addition of 5 mM-sodium pyrophosphate or boiled rat-liver supernatant (100 mg. equivalent of liver).

Accelerating effect of boiled supernatant on the conversion of L-*gulonolactone into* L-*ascorbic acid by rat-liver microsomes.* Boiled rat-liver supernatant has been found to accelerate to a great extent the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes. Boiled supernatant from rat liver, however, is not specifically active; boiled supernatants obtained from goat liver and chick kidney, as well as from guinea-pig liver, are also similarly active (Table 3).

Species difference in the requirement for chelating agent and boiled supernatant. It has been observed that, unlike rat-liver microsomes, the synthesis of ascorbic acid by goat-liver microsomes is not accelerated in the presence of any of the above-mentioned chelating agents or boiled supernatant (Fig. 4). The microsomes from chick kidney behave in a similar way to rat-liver microsomes.

Effect of heavy-metal ions on the conversion of L-*gulonolactone into* L-*ascorbic acid.* Results showing the effect of some added heavy-metal ions on the synthesis of ascorbic acid from L-gulonolactone are given in Table 4. The heavy-metal ions inhibit the synthesis probably by blocking the essential thiol groups involved in the synthesis (Chatterjee *et al.* 1958*c*).

Effect of thiol reagents. The rate of conversion of L-gulonolactone into L-ascorbic acid is strongly inhibited by *p*-chloromercuribenzoate, the inhibition

Table 4. *Effect of some heavy-metal ions on the conversion of* L-*gulonolactone into* L-*ascorbic acid by microsomes from different species*

Conditions are given in the Methods section.

Microsomes	Inhibition (%) Hg^{2+} (0·5 mM)	Cu^{2+} (0·5 mM)	Zn^{2+} (1·0 mM)
Rat liver	85	98	75
Goat liver	80	95	70
Chick kidney	82	96	73

Table 5. *Effect of thiol reagents on the conversion of* L-*gulonolactone into* L-*ascorbic acid by microsomes from different species*

Reduced glutathione (GSH) was added 10 min. after *p*-chloromercuribenzoate (PCMB). Conditions are described in the Methods section.

Microsomes	Inhibition (%) PCMB (0·5 mM)	PCMB (0·5 mM) with GSH (5 mM)
Rat liver	90	15
Goat liver	80	10
Chick kidney	85	10

being reversible with reduced glutathione. The results are given in Table 5.

It will be seen from Tables 4 and 5 that microsomes from goat liver and chick kidney behave in a similar manner to rat-liver microsomes in the inhibition by heavy-metal ions and by *p*-chloromercuribenzoate.

Solubilization and purification of the microsomal enzyme. Treatment of the microsomes with sonic or ultrasonic oscillations, irradiation with ultraviolet light, precipitation with protamine sulphate or treatment with digitonin or ribonuclease did not give fruitful results. Treatment with 20% ethanol in the presence of 0·5M-sodium chloride or incubation with pancreatic lipase resulted in significant loss in activity. The microsomes could, however, be effectively solubilized by treatment with either snake venom (cobra, *Naja naja*) or sodium deoxycholate. The use of deoxycholate for the solubilization of the microsomal enzyme has also been mentioned by Mapson (1958). The solubilized microsomes could be further purified by precipitation with ammonium sulphate, the details of which are described elsewhere (Chatterjee *et al.* 1959*b*). The enzyme, thus purified, appears to be a soluble one. After being centrifuged at 100 000 *g* for 1 hr. almost the entire activity of the original microsomes was found to reside in the supernatant.

Nature of the cofactor involved in the oxidation of L-*gulonolactone into* L-*ascorbic acid.* When rat-liver microsomes were treated with acid ammonium sulphate (Warburg & Christian, 1938), flavin was split off in the supernatant and the activity of the precipitated fraction in the conversion of L-gulonolactone into L-ascorbic acid was completely destroyed, but the activity was not restored by the addition of FAD or FMN. The soluble-enzyme preparation (30-fold concentrated), obtained by treatment with sodium deoxycholate followed by fractionation with ammonium sulphate, has been found to contain flavin (Chatterjee *et al.* 1959*b*). It was inhibited by Antabuse (disulfiram), riboflavin and a specific flavin inhibitor such as flavin monosulphate (Egami & Yagi, 1956) (Table 6). Even after treatment with active charcoal or dialysis against phosphate buffer for 3 hr. with constant stirring, the purified enzyme preparation retained its full activity and converted L-gulonolactone into L-ascorbic acid in the absence of any added cofactor. The results indicate that some flavin, rather strongly bound with the protein moiety of the enzyme, is probably involved in the oxidation of L-gulonolactone into L-ascorbic acid.

Specificity of the lactone structure of the precursor in the synthesis of ascorbic acid. Fig. 5 shows that, irrespective of the species, the supernatant inhibits the microsomal conversion of L-gulonolactone into L-ascorbic acid. This is apparently due to the presence of gulonolactonase in the supernatant, an enzyme fairly stable at 50°, which hydrolyses the lactone into L-gulonic acid (Chatterjee *et al.* 1959*a*), as will be evident from Fig. 7. The amount of gulonolactone (9·6 μmoles), which had disappeared at the end of incubation for 2 hr. by the action of gulonolactonase (Fig. 7), presumably by conversion into gulonate, was recovered by non-enzymic lactonization in the presence of dilute hydrochloric acid and estimated as described above. Fig. 6 shows that L-gulonate is not converted into L-ascorbic acid by the microsomal enzyme but is so converted after the addition of a preparation of gulonolactonase, obtained from the soluble supernatant from rat liver, to the system. Fig. 7 shows the corresponding amount of L-gulonolactone formed from gulonate under these conditions, the action of gulonolactonase being reversible. It will be observed from Figs. 6 and 7 that the rate of formation of gulonolactone can account for the rate of conversion of

Table 6. *Effect of flavin inhibitors on the conversion of* L-*gulonolactone into* L-*ascorbic acid by a soluble enzyme system from goat-liver microsomes*

The system contains 3 mg. protein equivalent of a soluble enzyme preparation (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*b*). Other conditions are given in the Methods section.

Inhibitor	Concn. (mM)	Inhibition (%)
Disulfiram	1	50
Riboflavin	0·5	40
Flavin monosulphate	0·01	25

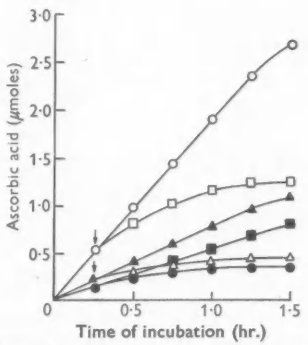

Fig. 5. Effect of soluble supernatant from rat liver on the rate of microsomal conversion of L-gulonolactone into L-ascorbic acid. Conditions are given in the Methods section. ○, Goat-liver microsomes; □, after addition of soluble supernatant. ▲, Chick-kidney microsomes; △, after addition of soluble supernatant. ■, Rat-liver microsomes; ●, after addition of soluble supernatant. Arrows indicate addition of soluble supernatant from rat liver (100 mg. equivalent of liver).

L-gulonate into L-ascorbic acid in the presence of the gulonolactonase. The conversion of L-gulonate into L-gulonolactone to a small extent in the presence of gulonolactonase, with the consequent formation of a small amount of ascorbic acid, is due to the reversible action of the enzyme, the equilibrium being more favourable towards the hydrolysis of the lactone.

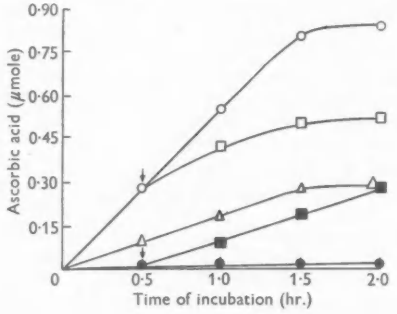

Fig. 6. Rate of formation of L-ascorbic acid from L-gulonolactone by rat-liver microsomes in the presence of gulonolactonase. Conditions are given in the Methods section. ○, With 5 mM-L-gulonolactone; □, after addition of gulonolactonase; △, with mM-L-gulonolactone; ●, with 5 mM-sodium L-gulonate; ■, after addition of gulonolactonase. Arrows indicate addition of gulonolactonase (1 mg. equivalent of protein).

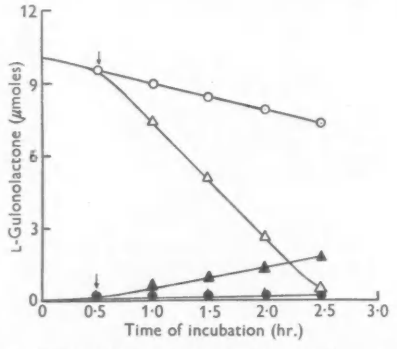

Fig. 7. Effect of gulonolactonase from rat liver on the formation and disappearance of lactone from sodium L-gulonate and L-gulonolactone respectively. With gulonate, neutral 0·4M-hydroxylamine was used as a trapping agent. Other conditions are given in the Methods section. ○, With 10 μmoles of L-gulonolactone; △, after addition of gulonolactonase; ●, with 10 μmoles of sodium L-gulonate; ▲, after addition of gulonolactonase. Arrows indicate addition of gulonolactonase (1 mg. equivalent of protein).

Gulonolactonase from different species. The gulonolactonase does not occur specifically only in the soluble supernatant from rat liver. It is also present in goat-liver and chick-kidney supernatants as well as in guinea-pig-liver supernatants (Table 7).

Effect of heat on gulonolactonase activity. Table 8 shows the effect of heat on the activity of gulonolactonase in the rat-liver supernatant. The activity is expressed in terms of μmoles of L-gulonolactone formed from 10 mM-sodium L-gulonate after an incubation period of 2 hr.

Relationship between syntheses of L-ascorbic acid and L-xylulose. The L-gulonic dehydrogenase preparation catalysed the reduction of added DPN when L-gulonate was used as the substrate, leading to the formation of L-xylulose, as will be observed in Figs. 8 and 9. The figures also show that, unlike L-gulonic dehydrogenase, neither the soluble microsomal-enzyme preparation nor the gulonolactonase, separately, or conjointly, could catalyse this reduction of added DPN leading to the formation of xylulose from L-gulonate.

The microsomes could convert L-gulonolactone into L-ascorbic acid without any added factor. L-Gulonate, however, was converted only when it was lactonized by prior incubation with gulonolactonase. In the latter case also no added cofactor was needed and, except L-ascorbic acid, no other

Table 7. *Effect of gulonolactonase obtained from different species on the synthesis of L-ascorbic acid from sodium L-gulonate by goat-liver microsomes*

Each flask contains 1 mg. protein equivalent of gulonolactonase, 10 μmoles of sodium gulonate and 0·25 ml. of goat-liver microsomes. Other conditions are given in the Methods section.

Source of gulonolactonase	Ascorbic acid synthesized (μmole)
Rat-liver supernatant	0·66
Goat-liver supernatant	0·55
Chick-kidney supernatant	0·60
Guinea-pig-liver supernatant	0·27

Table 8. *Effect of heat on the activity of gulonolactonase of rat-liver supernatant*

Figures in parentheses indicate the times for which the enzyme was kept at the corresponding temperature. Each flask contained 10 μmoles of sodium gulonate and 0·4M-hydroxylamine (neutral). Other conditions are given in the Methods section.

Temperature	Gulonolactone formed (μmoles)
37° (30 min.)	2·50
40 (30 min.)	2·45
50 (30 min.)	2·40
60 (15 min.)	1·20
70 (10 min.)	0·01

product of reaction was noticed, even in the presence of added DPN. The preparation of gulonolactonase was thus free from contamination with L-gulonic dehydrogenase. Whereas L-gulonate was converted into L-ascorbic acid by the microsomes in the presence of gulonolactonase and, in the absence of any added cofactor, it was not converted into L-xylulose by the L-gulonic dehydrogenase unless DPN was added to the system (Table 9). It is clear from the results given in Table 9 that the conversion of L-gulonate into L-ascorbic acid is a quite different and independent pathway from that of L-gulonate into L-xylulose. The former takes place via the formation of L-gulonolactone by the gulonolactonase and the latter via the hypothetical 3-keto-L-gulonate by the DPN-specific L-gulonic dehydrogenase.

Microsomes could not convert L-gulonolactone into L-xylulose (Table 9). This would indicate that the intermediate product in the microsomal oxidation of L-gulonolactone to L-ascorbic acid is most probably L-*xylo*hexulonolactone (2-keto-L-gulonolactone); 2-keto-L-gulonate could not be decarboxylated into L-xylulose (Table 9). That the product of oxidation might be the 3-keto compound, which, owing to the absence of a necessary decarboxylating enzyme in the microsomes is not decarboxylated, is unlikely in view of the fact that even after addition of a partially purified soluble supernatant containing the decarboxylase no formation of L-xylulose was detected (Table 9).

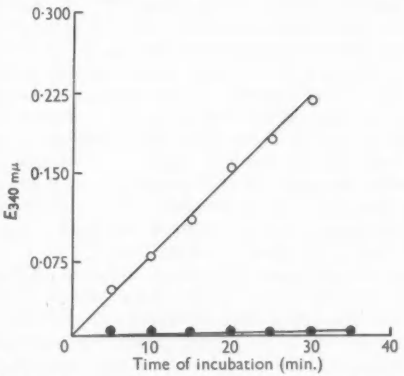

Fig. 8. Rate of reduction of DPN by L-gulonic dehydrogenase. ○, L-Gulonic dehydrogenase; ●, gulonolactone or soluble microsomal enzyme preparation added separately or conjointly. Each cuvette (1 cm.) contained 40 mM-sodium phosphate buffer, pH 7·8, 0·5 μmole of DPN, 10 μmoles of sodium L-gulonate, 4 mg. protein equivalent of L-gulonic dehydrogenase or 1 mg. protein equivalent of gulonolactonase obtained from rat liver or 5 mg. protein equivalent of the soluble microsomal enzyme, incubated at 30°. Total volume was 3 ml.

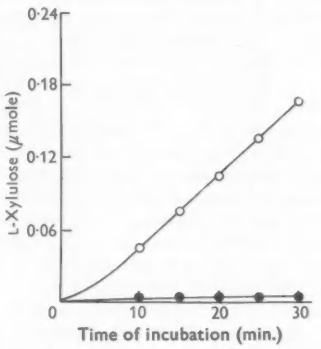

Fig. 9. Rate of synthesis of L-xylulose by L-gulonic dehydrogenase. ○, L-Gulonic dehydrogenase; ●, gulonolactonase or soluble microsomal enzyme preparation added separately or conjointly. Other conditions are the same as described for Fig. 8.

Table 9. *Relative conversion of* L-*gulonolactone and sodium* L-*gulonate into* L-*ascorbic acid*

The system contained 40 mM-sodium phosphate buffer, pH 7·4, 10 μmoles of substrate, 1·5 μmoles of DPN, 1 mg. protein equivalent of gulonolactonase (rat liver) or 4 mg. protein equivalent of soluble supernatant (obtained after fractionation with ammonium sulphate at 30–80% saturation of the salt at pH 7·8), 5 mg. protein equivalent of soluble microsomal enzyme (goat liver); incubated at 37° in air for 2 hr.; total volume 2·5 ml.

Source of enzyme	Substrate	Addition	L-Ascorbic acid formed (μmoles)	DPNH formed (μmole)	L-Xylulose formed (μmole)
Microsomes	L-Gulonolactone	—	2·50	—	Nil
		DPN	2·50	Nil	Nil
	L-Gulonate	—	Nil	—	Nil
		DPN	Nil	Nil	Nil
Microsomes and gulonolactonase		—	0·65	—	Nil
		DPN	0·65	Nil	Nil
		—	0·65	—	Nil
Microsomes and soluble supernatant		DPN	0·50	0·70	0·40
	L-Gulonolactone	—	1·0	—	Nil
	2-Keto-L-gulonate	—	—	—	Nil

Missing link in the guinea pig. Guinea-pig liver is known to contain the enzyme system converting D-glucuronic acid into L-gulonic acid (Hill & Chaikoff, 1956; Burns, 1957) as well as L-gulonic acid into L-xylulose (Touster *et al.* 1955; Ishikawa & Noguchi, 1957). As stated before, it has also been observed to contain the necessary gulonolactonase converting L-gulonate into the corresponding lactone. But it could not convert L-gulonolactone into L-ascorbic acid even in the presence of boiled supernatant or any of the above-mentioned chelating agents. This indicates that the biochemical step missing in the guinea-pig liver is the oxidation of L-gulonolactone to L-ascorbic acid. The presence of an inhibitor in the guinea-pig liver microsomes is excluded by the fact that when these microsomes were added to rat-liver microsomes no inhibition of the rate of conversion of L-gulonolactone into L-ascorbic acid was observed. Guinea-pig liver has, however, been found to contain the heat-stable factor which accelerates the conversion of gulonolactone into ascorbic acid (Table 3).

DISCUSSION

The enzyme catalysing the conversion of L-gulonolactone into L-ascorbic acid has been found to be located entirely in the microsomal fractions of the liver homogenates of rat and goat (Chatterjee *et al.* 1958*a*). It has also now been found to be located in the microsomal fraction of the kidney homogenate of chick (Table 1). The synthesis from rat-liver microsomes is accelerated in the presence of boiled supernatant or chelating agents such as sodium pyrophosphate, $\alpha\alpha'$-dipyridyl and 8-hydroxyquinoline. Boiled supernatant or chelating agent, however, has no influence on the synthesis by goat-liver microsomes. The possibility that boiled supernatant and chelating agents act by protecting the synthesized ascorbic acid from oxidative breakdown has been eliminated by the fact that all of the ascorbic acid (2 μmoles) added to the system could be recovered in the absence of boiled supernatant or chelating agent. The metal-binding agents might act by chelating some metal [e.g. Zn^{2+}, present in the rat-liver microsomes (Thiers & Vallee, 1957)], which has been found to be inhibitory to the conversion of L-gulonolactone into L-ascorbic acid (Table 4). The action of the boiled supernatant might also be due to some chelating agent present in it. The mode of action of these factors and the reason for the difference in the behaviour between rat- and goat-liver microsomes in the requirement for these factors are under further investigation.

The synthesis of ascorbic acid from L-gulonolactone has been found to be inhibited by heavy-metal ions and by *p*-chloromercuribenzoate, the inhibition by the latter being reversible by reduced glutathione, indicating that some essential thiol groups are involved in this reaction.

The conversion of L-gulonolactone into L-ascorbic acid by the microsomal enzyme requires the presence of oxygen (Chatterjee *et al.* 1957*b*; 1958*a*, *b*) but is not inhibited by 2 mM-potassium cyanide, indicating that a cytochrome system is not involved in this oxidation.

The oxidation of L-gulonolactone to L-ascorbic acid is significantly inhibited by flavin inhibitors such as disulfiram, riboflavin and flavin monosulphate. The partially purified soluble enzyme contains flavin (Chatterjee *et al.* 1959*b*); it retains its full activity, without any added factor, even after prolonged dialysis. The oxidation of L-gulonolactone to L-ascorbic acid is thus probably mediated by a thiol-containing flavoprotein, the flavin part of which is rather firmly bound with the protein moiety of the enzyme. This confirms the earlier report from this Laboratory (Chatterjee *et al.* 1957*b*, 1958*b*) that a flavoprotein might be involved in the oxidation of L-gulonolactone to L-ascorbic acid. The involvement of a flavoprotein in the conversion of L-galactonolactone into L-ascorbic acid by plant systems has been indicated by Mapson & Breslow (1958).

The microsomes have been found to utilize L-gulonolactone and not the sodium salt of the corresponding free acid. This failure to metabolize the free acid may be due to difficulties of penetration of the ionized compound through the microsomal lipoid barrier, since it is known that polar compounds generally do not penetrate the microsomes whereas non-polar compounds do (Mitoma, Posner, Reitz & Udenfriend, 1956). But this explanation appears improbable in view of the fact that a preparation of soluble enzyme from the microsomes still catalyses the oxidation of L-gulonolactone and not of sodium L-gulonate. The free acid can, however, be converted into ascorbic acid by the microsomal enzyme if it is lactonized beforehand by incubation with gulonolactonase, an enzyme present in the soluble supernatant, which reversibly converts the lactone into free acid. The synthesis of L-ascorbic acid then runs parallel with the formation of L-gulonolactone, the latter having been identified by paper chromatography. The rate of synthesis of ascorbic acid from L-gulonolactone in the presence of microsomes alone is about four to five times that obtained from gulonate in the presence of microsomes plus gulonolactonase. The action of gulonolactonase is reversible, the equilibrium being more favourable towards the hydrolysis of the lactone. The conversion of L-gulonate into L-gulonolactone is therefore the rate-limiting step in the synthesis. With L-gulonolactone as the substrate, addition of gulonolactonase to the microsomes inhibits the synthesis to a great extent but

the rate is still significantly higher than that obtained with L-gulonate. Contrary to the postulation of Grollman & Lehninger (1957) and of Bublitz, Grollman & Lehninger (1958) that the free gulonic acid is the precursor of ascorbic acid and that the lactone is acted on only after hydrolysis, it is clear that the lactone is the precursor of ascorbic acid and that the free acid is acted on only after lactonization. This confirms earlier observations from this Laboratory (Chatterjee *et al.* 1957*a*, *b*; 1958*a*, *b*; 1959*a*). Moreover, it has been observed, with L-gulonate as the substrate, that, unlike the soluble supernatant, microsomes alone or microsomes in conjunction with gulonolactonase do not catalyse the reduction of added DPN and also no xylulose is formed. This indicates that in the presence of soluble supernatant containing L-gulonic dehydrogenase and gulonolactonase the microsomal formation of ascorbic acid from L-gulonate depends upon the amount of L-gulonolactone formed by the action of gulonolactonase and not on the hypothetical 3-ketogulonate formed by the action of L-gulonic dehydrogenase. Hence the synthesis of L-ascorbic acid is a process competitive with the formation of L-xylulose at the stage of oxidation of L-gulonate by the DPN-specific L-gulonic dehydrogenase and not with the subsequent decarboxylation of the hypothetical '3-keto-L-gulonate'. Since one-step oxidation of gulonolactone would lead to the formation of ketogulonolactone and since ketogulonolactone is non-enzymically converted into ascorbic acid, there is no necessity to postulate, as has been done by Grollman & Lehninger (1957), the presence of a specific lactonizing enzyme in the microsomes converting 3-keto-L-gulonic acid into the corresponding lactone.

When, instead of L-gulonate, D-glucuronate is used as the substrate, addition of gulonolactonase to the microsomal system does not lead to the formation of ascorbic acid. In this case addition of soluble supernatant and TPNH is necessary for the synthesis. Soluble supernatant is known to contain a TPN-dependent aldehyde dehydrogenase (Hers, 1956; Hassan & Lehninger, 1956) which reduces D-glucuronic acid probably to L-gulonate, though it has not so far been identified. L-Gulonate is then lactonized to L-gulonolactone by the gulonolactonase of the supernatant, and the latter by the action of the microsomal enzyme is converted into L-ascorbic acid. The formation of ascorbic acid from D-glucuronate is thus limited by two rate-determining steps, namely D-glucuronate to L-gulonate and L-gulonate to L-gulonolactone. Actually it has been found in this Laboratory that the rate of formation of ascorbic acid from D-glucuronate in the presence of soluble supernatant plus microsomes is about one-sixth of that obtained from the lactone in the presence of microsomes alone.

Taking into consideration the above described facts, the sequences of reactions involved in the biosynthesis of L-ascorbic acid as well as of L-xylulose may be represented by the scheme shown in Fig. 10.

Thus there is a remarkable difference between the enzymic oxidation of L-gulonate and that of L-gulonolactone. With the former, the free acid is the substrate and the oxidation is catalysed by the DPN-specific L-gulonic dehydrogenase of the soluble supernatant, and addition of DPN is necessary; whereas, with the latter, the lactone is the substrate and the enzyme, very probably a flavoprotein, is located entirely in the microsomes and no addition of cofactor is needed. With gulonate the product of oxidation should be sufficiently unstable to be decarboxylated into L-xylulose and has usually been considered to be 3-keto-L-gulonate. But in ascorbic acid formation the intermediate product should be rather stable in order to maintain its C_6 chain, and there is every possibility of 2-keto-L-gulonolactone being this intermediate. 2-Keto-L-gulonate has not been found to lead to the formation of L-xylulose (Table 9), showing that the former is not decarboxylated by the enzyme of the soluble supernatant, an observation which has also been made by Burns, Kanfer & Dayton (1958) using [^{14}C]2-keto-L-gulonate. Further, even after addition to the system containing microsomes and L-gulonolactone, of a partially purified soluble supernatant

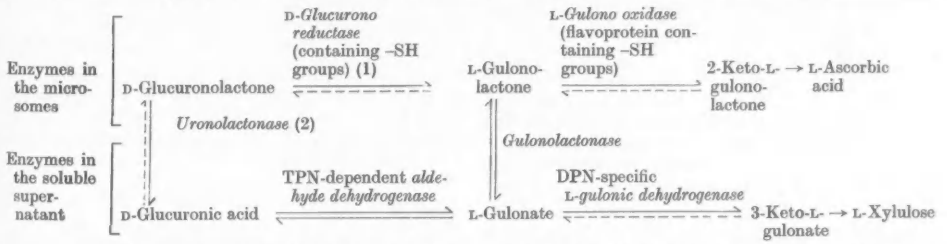

Fig. 10. Schematic representation of the pathways of biosynthesis of L-ascorbic acid and of L-xylulose. (1) Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1958*c*). (2) Winkelman & Lehninger (1958). - - - - - -, Reversibility not known.

containing the decarboxylating enzyme, no detectable formation of L-xylulose was noticed (Table 9). Had the 3-keto compound been the intermediary in the microsomal oxidation of L-gulonolactone to L-ascorbic acid, the formation of L-xylulose in the above system could have been expected.

In species requiring dietary ascorbic acid, e.g. the guinea pig, the liver enzyme has been found to convert D-glucuronic acid into L-gulonic acid (Hill & Chaikoff, 1956; Burns, 1957). It has also been observed to contain the necessary lactone-forming enzyme converting L-gulonate into L-gulonolactone. But L-gulono-oxidase, the microsomal enzyme catalysing the oxidation of L-gulonolactone into L-ascorbic acid, is absent. This missing biochemical step is perhaps a general characteristic of all the species which are unable to synthesize L-ascorbic acid.

SUMMARY

1. The enzyme catalysing the conversion of L-gulonolactone into L-ascorbic acid has been found in the liver microsomes of the rat and the goat and kidney microsomes of the chick.

2. The synthesis of ascorbic acid from gulonolactone by rat-liver and chick-kidney microsomes is accelerated by the metal-binding agents sodium pyrophosphate, $\alpha\alpha'$-dipyridyl and 8-hydroxyquinoline and by the boiled supernatant from livers of rat, goat and guinea pig and kidney of the chick. These factors do not accelerate the synthesis of ascorbic acid by goat-liver microsomes.

3. The enzyme converting L-gulonolactone into L-ascorbic acid is inhibited by heavy-metal ions (Hg^{2+}, Cu^{2+}, Zn^{2+}) and by *p*-chloromercuribenzoate, the inhibition by the last-named being reversible with reduced glutathione, indicating the involvement of thiol groups in the enzyme.

4. The conversion of L-gulonolactone into L-ascorbic acid by a soluble and purified preparation of the microsomal enzyme is inhibited by the flavin inhibitors riboflavin, Antabuse (disulfiram) and riboflavin monosulphate, indicating the involvement of a flavoprotein.

5. With sodium L-gulonate as substrate the soluble enzyme preparation does not reduce diphosphopyridine nucleotide and no xylulose formation was detected.

6. L-Gulonolactone and not L-gulonate is the precursor of ascorbic acid. Gulonate is converted into ascorbic acid only after lactonization by the action of gulonolactonase, an enzyme present in the soluble supernatant. The action is reversible and the equilibrium favours hydrolysis of the lactone.

7. A scheme showing the pathways of the biosyntheses of L-ascorbic acid and of L-xylulose is given. The conversion of L-gulonolactone into L-ascorbic acid is shown to follow a different and independent pathway from that of L-gulonolactone into L-xylulose. The former is catalysed by an enzyme present entirely in the microsomes and the latter by an enzyme system present only in the soluble supernatant.

8. The possibility of L-*xylo*hexulonolactone (2-keto-L-gulonolactone) being an intermediate in the synthesis of L-ascorbic acid is discussed.

9. In guinea-pig liver, the microsomal enzyme catalysing the conversion of L-gulonolactone into L-ascorbic acid is missing.

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The Determination of Blood Glutathione

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The nitroprusside method for estimating glutathione is non-specific and suffers from the defect of an unstable colour, although recent improvements (Thompson & Watson, 1952; Grunert & Phillips, 1951) appear to make the method more satisfactory. Binkley, Fufii & Kimmell (1950) used a method based on the Sullivan & Hess (1936) reaction to estimate glutathione, γ-glutamylcysteine, cysteinylglycine and cysteine, but in our hands it failed to give reproducible results. We therefore had to seek another method and investigated the observation by Patterson, Lazarow & Levey (1949) that alloxan and glutathione interact to form a compound characterized by an absorption band at 305 mμ. Other sulphydryl compounds, e.g. cysteine, react with alloxan but produce dialuric acid by reduction. This last-named substance has an absorption maximum at 275 mμ and does not interfere with measurements at 305 mμ. Some proteins react with alloxan but these can be removed by precipitation, as in the proposed method.

It appeared possible therefore that the reaction with alloxan could be used to estimate glutathione in blood, thus avoiding the interference of the peptides cysteinylglycine and γ-glutamylcysteine, which, present in blood, react in other methods as glutathione. A very sensitive method, using this reaction, has been developed which readily detects 15 μg. of glutathione and can be used to estimate it in as little as 0·2 ml. of blood. We have not attempted to extend the method to the estimation of glutathione in tissues. The method was used to investigate the blood-glutathione concentration in some cases of therapeutic deep insulin coma.

METHOD

Principle. Whole lysed blood is deproteinized with trichloroacetic acid, neutralized with buffered NaOH and alloxan is added to develop the compound with glutathione (GSH), which has a characteristic absorption at 305 mμ.

Reagents. 10% (w/v) Trichloroacetic acid.

0·24 M-Phosphate buffer, pH 7·6, prepared by mixing 0·24 M-Na_2HPO_4 with 0·24 M-KH_2PO_4 (88:12, v/v).

Neutralizing NaOH: this contains in 1·5 ml. of solution both 0·24 M-phosphate buffer and enough N-NaOH to neutralize 0·5 ml. of supernatant from protein precipitation (219:21, v/v).

Alloxan reagent: 1 mg. of alloxan monohydrate/ml. in dilute HCl (1 drop of conc. HCl/100 ml. of water). Prepared freshly and used within 5 min.

Standard GSH: 10 mg./100 ml. of water, freshly prepared.

0·1 M-Glycine solution.

Procedure. A specimen (0·2 ml.) of whole blood from a finger prick is added to 0·6 ml. of water. After it has been allowed to stand for 10 min. to haemolyse, 0·8 ml. of trichloroacetic acid is added, the liquids are mixed immediately with a glass rod and centrifuged for 5 min.

For each sample of blood two tubes are prepared: test (tube 1) and control (tube 2); each contains 0·5 ml. of glycine solution, and 2·9 and 3·9 ml. of phosphate buffer are added to (1) and (2) respectively. To each tube 0·4 ml. of supernatant from the deproteinized blood and then 1·2 ml. of neutralizing NaOH are added, with mixing.

An alloxan blank (tube 3: 0·5 ml. of glycine solution and 4·5 ml. of phosphate buffer), a phosphate blank (tube 4: 6 ml. of phosphate buffer) and a standard tube (tube 5: 0·5 ml. of glycine solution, 4·3 ml. of phosphate buffer and 0·2 ml. of standard glutathione) are also prepared.

Fresh alloxan solution (1 ml.) is added to tubes 1 (test), 3 (alloxan blank) and 5 (standard) and the contents of each tube are mixed. The final volume in each tube is 6 ml. This procedure is summarized in Table 1.

Readings. All extinctions are read in 2 cm. silica cells in a spectrophotometer set at 305 mμ. (A Uvispek spectrophotometer was used with hydrogen-arc lamp and slit width 19 = 0·38 mm., waveband width 5 mμ.) The control tube 2 is read against the phosphate blank immediately, as its absorption at 305 mμ increases slightly on standing. For completion of the reaction 20 min. is allowed and the test tube 1 and standard tube 5 are then read against the alloxan blank (tube 3).

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Table 1. *Preparation of tubes for estimation of glutathione*

Material	Vol. of material added (ml.) Tube 1 Test	Tube 2 Control	Tube 3 Alloxan blank	Tube 4 Phosphate blank	Tube 5 Standard
Glycine	0·5	0·5	0·5	—	0·5
Phosphate buffer	2·9	3·9	4·5	6·0	4·3
Deproteinized blood	0·4	0·4	—	—	—
Neutralizing NaOH	1·2	1·2	—	—	—
Standard GSH	—	—	—	—	0·2
Alloxan	1·0	—	1·0	—	1·0

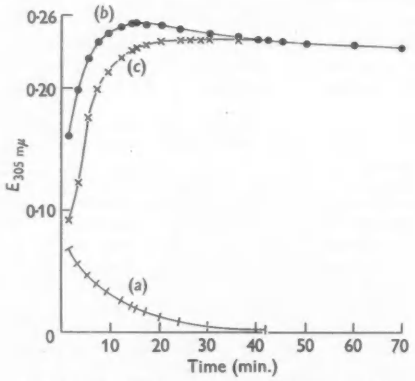

Fig. 1. 'Colour' development curves of reaction between glutathione and 1 mg. of alloxan in the presence of 5 μg. of cysteine and 0·5 ml. of 0·1 M-glycine, in a reaction mixture of 6 ml. Readings of alloxan blank (*a*) and glutathione standard (*b*) were made against phosphate buffer. Standard less blank readings are indicated by (*c*).

The extinction of the alloxan blank rapidly decreases, whereas that of the standard increases, during the first 20 min. The maximum difference occurs between 20 and 40 min., during which time results are constant (Fig. 1).

Calculation. Let control reading against phosphate blank = E_A, test reading against alloxan blank = E_B, standard reading against alloxan blank = E_S. Then

$$40\,(E_B - E_A)/E_S = \text{mg. of GSH/100 ml. of blood.}$$

RESULTS

The results of estimating GSH in blood from 14 normal adults (seven male, seven female) are given in Table 2. These values represent only GSH since oxidized glutathione does not react with alloxan (Patterson *et al.* 1949). The range of values is 24·4–37·3 mg./100 ml. and the mean 30·0, S.D. ± 4·0. The mean value for males was 31·6 mg./100 ml., S.D. ± 4·4, and for females 28·4 mg./100 ml., S.D. ± 2·9. The difference between these means is not statistically significant ($P < 0·2 > 0·1$). A sex difference does not seem probable, but it would be unsafe to draw a firm conclusion from such a small series. When corrected by a recovery factor of 86 % (see below), the range of values becomes 28·4–43·4 mg./100 ml. and the mean 34·8 mg./100 ml.

Table 2. *Concentration of glutathione in normal adult blood*

Concn. (mg./100 ml.) Males	Females
24·4	25·4
26·5	25·8
31·2	26·1
31·6	27·6
34·6	29·7
35·4	30·3
37·3	34·0
Mean 31·6	28·4
S.D. ± 4·4	± 2·9

All 14: mean 30·0, S.D. ± 4·0.

Recovery experiments. In a series of recovery experiments in which known amounts of GSH were added to blood after lysis, 78–93 % (mean 86 %, S.D. ± 4) of the added GSH was accounted for. The result of one experiment is given in Table 3.

The reasons for the low variable recovery were not explored. It may be due to oxidation of GSH added to blood or to interfering substances in the supernatant after protein precipitation. Until the reasons for the low recovery are settled, the question of the advantage of using added GSH as an internal standard in the method is left open. If the low recovery is due to the oxidation of GSH added to blood, it seems likely that the proportion recovered represents the proportion of the total glutathione in blood present as GSH.

Effect of pH. The effect of various phosphate buffer mixtures is shown in Fig. 2, from which it is evident that the buffer mixture chosen gives maximum formation of the GSH–alloxan compound as measured by extinction.

Since the reaction is sensitive to pH changes, great care is necessary in the preparation and measurement of the neutralizing solution. Incomplete neutralization in particular gives rise to

Table 3. *Recovery of glutathione added to blood after lysis*

All values are given as mg. of glutathione/100 ml.

Blood GSH	GSH added	Theoretical value	GSH found	GSH recovered
26·9	11·66	38·56	37·8	10·9
26·9	23·32	50·22	46·9	20·0
26·9	34·98	61·88	56·9	30·0
	69·96			60·9 (= 87% of GSH added)

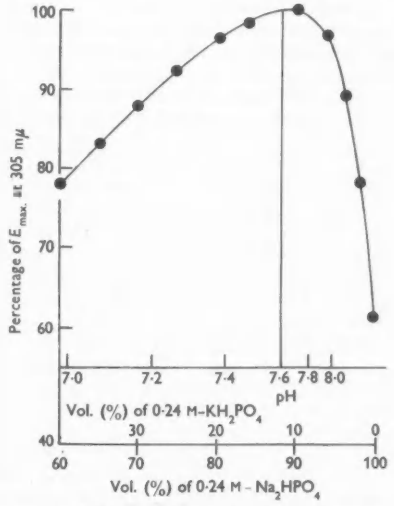

Fig. 2. Effect of various phosphate buffer mixtures on the GSH–alloxan reaction.

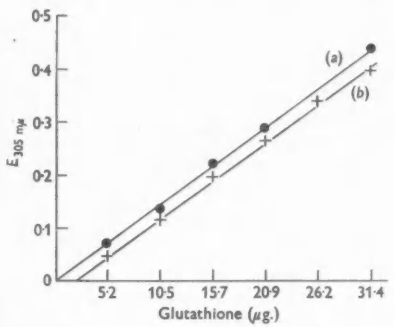

Fig. 3. Calibration curves for glutathione with 1 mg. of alloxan, showing the influence of the volume of the solution. (*a*) Reaction volume of 3 ml., to which 3 ml. of phosphate buffer was afterwards added; (*b*) reaction volume of 6 ml.

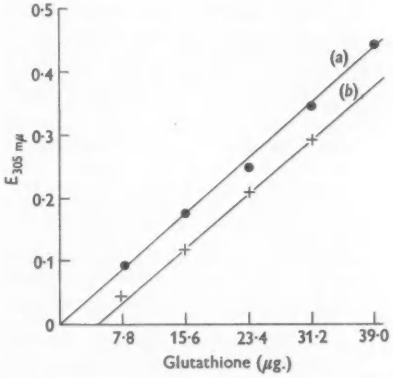

Fig. 4. The presence of 0·2 ml. of 0·1 M-glycine (*a*) improved the reaction between glutathione and 1 mg. of alloxan in 6 ml. of reaction mixture; (*b*) reaction without glycine.

errors, e.g. a deficiency of 0·05–0·1 ml. of the neutralizing solution may give errors of the order of −3 to −8%.

Effect of alloxan concentration. Calibration curves obtained from reaction mixtures of 3 ml. volume containing 1 mg. of alloxan show good proportionality between glutathione and extinction (Fig. 3*a*), but the same mixtures in 6 ml. with 1 mg. of alloxan gave a graph which did not intersect the origin (Fig. 3*b*). As would be expected, 6 ml. mixtures containing 2 mg. of alloxan produced a straight-line graph passing through the origin.

Effect of glycine. When 0·2 ml. of 0·1 M-glycine was added, 1 mg. of alloxan in a 6 ml. mixture gave a good calibration curve (Fig. 4*a*), comparable to that given when 2 mg. of alloxan was used without glycine in 6 ml. Fig. 4*b* was obtained simultaneously without glycine. Thus the addition of glycine has an effect similar to that given by doubling the concentration of alloxan, although it does not give the same extinction values. It has, however, the great advantage of giving a much lower extinction in the blank.

Activation and inhibition. It was recognized that certain factors in the solutions (possibly heavy-metal ions) might be responsible for the anomalous calibration curves obtained in the absence of glycine. A number of compounds known to be 'sequestering agents' for metal ions were therefore included in the reaction mixture. Sodium cyanide and sodium dithionite reacted with alloxan in a way that produced a large extinction even in the absence of GSH. Of the other reagents chosen (Table 4), glycine had the greatest effect, but from Table 5 it can be seen that other amino acids had a similar or greater effect. Glycine was selected for the method purely as a matter of convenience. The maximum effect of glycine was given when 0·5 ml. of 0·1 M-glycine was included in 6 ml. of reaction mixture. Glycine also improved the recovery values of added GSH in the method with blood.

β-Alanine and methionine (Table 5) and cysteine (Table 6 and Fig. 5) have inhibitory effects on the GSH–alloxan reaction. The mechanism of this was not investigated, but there seems to be a distinguishing feature in that, in the presence of glycine, methionine inhibition is not shown, whereas cysteine inhibition is unchanged (Fig. 5). In the absence of glycine, 2 μg. of either methionine or cysteine showed an appreciable inhibitory effect

Table 4. *Effect of 'sequestering agents' on the reaction between glutathione and alloxan*

GSH (20 μg.) was treated with 1 mg. of alloxan in 6 ml. of solution containing the sequestering agent (0·2 ml.; 0·1 M). Readings were made at 305 mμ against the appropriate blank.

Sequestering agent	*E* due to GSH
Nil	0·230
$Na_2S_2O_3$	0·231
Sodium citrate	0·237
Sodium tartrate	0·238
Glycine	0·280
NaCN	No reading possible because of high blank
$Na_2S_2O_4$	

Table 5. *Effect of various reagents on the reaction between glutathione and alloxan*

GSH (19·02 μg.) was treated with alloxan (2 mg.) in 6 ml. of solution containing the test reagent (0·3 ml.; 0·1 M). Readings were made against the appropriate blank at 305 mμ. The extinction readings of the blanks were all low, except for methionine (0·069 against phosphate blank).

Reagent	*E* due to GSH
Nil	0·285
Sodium citrate	0·300
Glycine	0·320
α-Alanine	0·322
Threonine	0·353
Aspartic acid	0·353
Serine	0·357
β-Alanine	0·271
Methionine	0·135

Table 6. *Inhibition by methionine and cysteine of the glutathione–alloxan reaction*

The reaction mixtures (6 ml.) contained alloxan (2 mg.) and, when present, GSH (19·02 μg.), cysteine (2 μg.) and methionine (2 μg.).

Reaction mixture	$E_{305\ m\mu}$
A GSH	0·293
GSH + cysteine	0·274
Cysteine	0·004
B GSH	0·289
GSH + methionine	0·262
Methionine	0·009

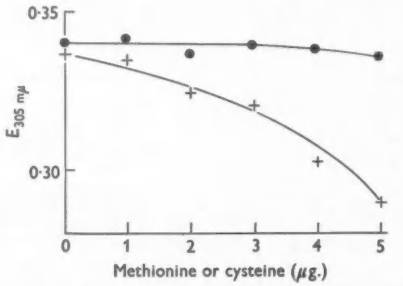

Fig. 5. Comparison of the effect of glycine (0·5 ml.; 0·1 M) on the inhibitive effects of methionine (●) and cysteine (+) on the interaction between 19·02 μg. of glutathione and 2 mg. of alloxan in 6 ml. of solution.

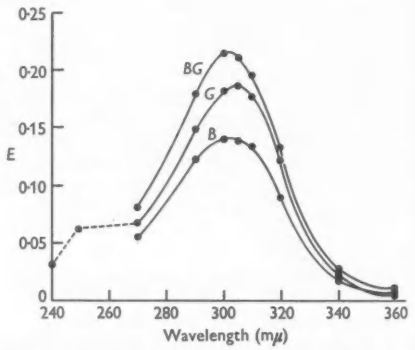

Fig. 6. Ultraviolet-absorption curves of the GSH–alloxan compound (*G*), the substance in blood reacting with alloxan (*B*) and of the alloxan compound formed in blood with added GSH (*BG*).

(Table 6), which could be important in some other applications of this method.

Specificity. The ultraviolet-absorption spectra of the compounds of alloxan with GSH (curve *G*), deproteinized blood (curve *B*) and deproteinized blood with added GSH (curve *BG*) are given in Fig. 6. The curves, especially *G*, are very similar to those given by Patterson *et al.* (1949). The molar extinction at 305 mμ, calculated from curve *G* on the basis of the substance in lowest concentration (GSH), is 17 130. This value is very close to the maximum value of 17 400 found by Patterson *et al.* In their case, alloxan and not GSH was the substance present in the lowest concentration. It is apparent that under the conditions of their and our experiments quantitatively similar formation of the alloxan–GSH compound occurs.

When blood supernatant was present (curves *B* and *BG*) the extinction due to the alloxan compound could not be measured at wavelengths below 270 mμ because of the high absorption by the neutralized supernatant. Although the absorption maxima of the alloxan compound with the reacting substance in blood supernatant (curves *B* and *BG*) occur at about 302 mμ and not at 305 mμ as with GSH, the curves *B* and *BG* are so nearly like that given by GSH (*G*) that it is reasonable to conclude that the substance from blood that reacts with alloxan is GSH.

The peptides cysteinylglycine and γ-glutamylcysteine in blood present the problem of possible interference by producing substances which may have absorption bands at or near 305 mμ. Because these compounds are not readily available, it has not been possible to prove the complete specificity of the method. Cysteinylglycine, however, is produced from GSH by controlled hydrolysis with hot 2M-phosphoric acid for 1 hr. (Binkley *et al.* 1950). After neutralization it was found that the alloxan-reacting material had been destroyed by the hydrolysis.

GSH (35 μg.) in 2M-phosphoric acid, neutralized and allowed to react with alloxan, gave an extinction at 305 mμ of 0·393. GSH (35 μg.) hydrolysed with hot 2M-phosphoric acid, neutralized and allowed to react with alloxan, gave an extinction at 305 mμ of 0·018. The fall in extinction, 0·375, due to hydrolysis of the alloxan-reacting material, corresponds to the extinction of 0·377 given by 35 μg. of GSH in the alloxan reaction. Thus if cysteinylglycine is formed by hydrolysis of GSH, it does not react with alloxan to give any absorption at 305 mμ and does not interfere with the estimation of GSH. Ergothioneine (29 μg.) and alloxan did not cause any absorption at 305 mμ.

Conclusion. The effect of various substances on the interaction between GSH and alloxan indicates that any method for estimating GSH using absorption at 305 mμ must exclude interference by substances occurring naturally in blood. The influence of glycine on the reaction appears similar to its effect on the interference by amino acids in the Nakamura & Binkley method (1948) for cysteine, recorded by Hird & Springell (1954), in which glycine eliminated the depression of colour production caused by certain other amino acids.

Blood glutathione in therapeutic deep insulin coma

A short investigation was made of the blood GSH concentration in therapeutic deep insulin coma. By the courtesy of Dr A. S. Thorley, in 1953 four patients were studied. They were adult males who had had eight to 14 comas previously. Blood was collected after onset of coma until recovery. The results are given in Table 7. Blood GSH concentration was not affected in these patients by the state of hypoglycaemic coma or by the amount of insulin given to induce coma. Patient 4 had a slightly subnormal blood GSH both during coma and after recovery when the blood glucose was in the normal range.

In prolonged hypoglycaemic coma, when a patient undergoing therapeutic deep insulin coma fails to recover after being given glucose, abnormal values of blood GSH may occur. In one patient values of 77–90 mg./100 ml. were found, the blood glucose being normal or above normal. In another, a value of 65 mg./100 ml. was obtained but in two others normal values (29·8, 31·2 and 33·9 mg./100 ml.) were found. There did not seem to be any simple relationship between the blood GSH and the prolonged coma.

Table 7. *Blood concentration of glutathione in therapeutic deep insulin coma*

Patient no.	Units of insulin given	No. of coma	Time (min.) after onset of coma: 7	30–40	60–70	100	After recovery
			Blood GSH (mg./100 ml.)				
1	320	12	34·8	34·4	35·7	34·4	—
2	280	9	—	36·2	37·1	36·8	34·3
3	220	15	—	30·6	31·5	30·0	—
4	120	10	—	23·8	23·4	23·1	22·3

SUMMARY

1. The application of the reaction between glutathione and alloxan, giving a substance characterized by an absorption band at 305 mμ, to the estimation of glutathione in blood is described.

2. Certain substances influence the reaction: β-alanine, methionine and cysteine are inhibitors and citrate, glycine and other α-amino acids are activators.

3. Some observations of blood glutathione concentration in normal and prolonged therapeutic deep insulin coma are recorded.

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